UNIVERSIDAD AUTÓNOMA DE MADRID
DEPARTAMENTO DE BIOQUÍMICA

FUNCIÓN DEL RECEPTOR X DE RETINOIDES EN LA REGULACIÓN TRANSCRIPCIONAL MEDIADA POR LOS RECEPTORES DE HORMONAS TIROIDEAS Y VITAMINA D3

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RESUMEN

En este trabajo hemos revisado el papel del receptor X de retinoides (RXR) y su ligando en los heterodímeros formados con los receptores de hormonas tiroideas (TR) y vitamina D (VDR), encontrando que no es un "compañero silencioso" para estos receptores sino que cumple importantes funciones transcripcionales dentro del heterodímero. Hemos encontrado que estos heterodímeros son capaces de reclutar coactivadores y activar la transcripción tanto de construcciones reporteras, como de genes diana (prolactina y 1α,25-dihidroxivitamina D3 24-hidroxilasa *cyp*24, para TR y VDR respectivamente), en respuesta a ambos ligandos de la pareja heterodimérica. El 9-*cis*RA coopera con los ligandos de sus parejas produciendo claros efectos aditivos y en algunos casos sinérgicos. La combinación con el rexinoide permite además recuperar la actividad del heterodímero en situaciones defectivas como es el caso de receptores o coactivadores mutados y de ligandos poco activos. Nuestros resultados indican que la magnitud de las respuestas transcripcionales a los rexinoides en los distintos tipos celulares está fuertemente influenciada por los niveles de coactivadores y de correpresores. Por otro lado, la combinación del ligando del RXR con dosis bajas de vitamina D o de agonistas parciales del VDR permite la diferenciación de células de cáncer de colon mediante el aumento de la expresión de E-Cadherina. En este trabajo hemos estudiado también la capacidad de reclutamiento de correpresores del heterodímero VDR/RXR. A diferencia de otros heterodímeros, éste recluta los correpresores SMRT y NCoR en respuesta a agonistas del VDR y los libera tras la unión del ligando del RXR. Puesto que la unión de la vitamina D también provoca reclutamiento de coactivadores, los correpresores actuarían como reguladores negativos de la respuesta transcripcional a vitamina D en los distintos tipos celulares, lo que se comprueba con el uso de mutantes específicos y con la inhibición de la expresión mediante siRNA de SMRT y NCoR. Todos estos hallazgos indican que las respuestas transcripcionales a los ligandos de estos receptores nucleares en los distintos tejidos vienen determinadas por el balance celular entre coactivadores y correpresores. Por último, hemos caracterizado varios análogos sintéticos de la vitamina D algunos de los cuales presentan efectos transcripcionales disociados. Estos compuestos apenas estimulan la transcripción, pero sin embargo son capaces de transrreprimir con similar potencia a la de la vitamina D. Su alta afinidad por el receptor y su escasa actividad calcémica les confieren un gran potencial terapéutico en distintas dolencias relacionadas con la función del VDR.

SUMMARY

In this work we have revised the role of the rexinoid X receptor (RXR) in the context of the heterodimers formed with the thyroid hormone (TR) and vitamin D (VDR) receptors. We have studied TR/RXR and VDR/RXR heterodimers using the rat prolactin gene and the 1α,25-dihydroxyvitamin D3 24-hydroxylase (*cyp*24) as model target genes. These heterodimers are able to recruit coactivators and stimulate transcription in response to the ligand of each receptor moiety. In addition, cooperative or synergistic effects are obtained when ligands of both partner receptors are present. Combination with 9-*cis*RA restores the activity of defective heterodimers such as those bearing mutated receptors or coactivators, and confers significant agonistic activity to otherwise inactive ligands. We found that transcriptional responses to rexinoids are strongly determined by the cellular levels of coactivators and corepressors. In addition, we have observed that the presence of 9-*cis*RA increases VDR-dependent differentiation of colon carcinoma cells stimulating the expression of E-Cadherin. We have also studied corepressors recruitment by VDR/RXR. Unlike other heterodimers of non-steroidal receptors, a VDR agonist-dependent recruitment of SMRT and NCoR to VDR/RXR was found. Moreover, RXR ligand binding releases corepressors bound in response to vitamin D. Since vitamin D binding also causes coactivators recruitment, corepressors could act as negative modulators of vitamin D-mediated transcriptional responses. This was proved with the use of specific mutants and by knock-down of SMRT and NCoR by means of siRNA. All these findings indicate that transcriptional responses to the different ligands of RXR heterodimers may be determined by the cellular ratio between coactivators and corepressors in the different target tissues. Finally we have characterized several vitamin D analogs some of which have a "dissociated" activity, they cannot transactivate but they are as potent as vitamin D in transrepression assays. Since they bind VDR with high affinity but are devoid of calcemic activity, they could have therapeutic utility in diseases in which VDR function is involved.

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Clave de Abreviaturas

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9-*cis*RA	ácido 9-*cis*- retinoico
ACTR	activador de los receptores de hormonas tiroideas y ácido retinoico
AD	dominio de activación
AF-1	función de activación 1 o función de activación independiente de ligando
AF-2	función de activación 2 o función de activación dependiente de ligando
AP-1	proteína activadora 1
AR	receptor de andrógenos
ARA70	activador 70 del receptor de andrógenos
atRA	ácido todo-*trans*- retinoico
BAFs	factores asociados a BRG-1
bHLH	dominio del tipo hélice-giro-hélice básico
BRG-1	gen relacionado con Brahma 1
CAR	receptor constitutivo de androstanos
CARM1	arginina metiltransferasa 1 asociada a cofactor
CBP	proteína de unión a CREB
CoCoA	coactivador "coiled coil"
CoR	correpresor
CoRNR	caja de unión a correpresores de los receptores nucleares
COUP	estimulador "upstream" de la ovoalbúmina de pollo
CREB	factor de unión a los elementos de respuesta a AMP cíclico
C-terminal	carboxilo terminal
Cyp24	1α,25-dihidroxivitamina D3 24-hidroxilasa
Cyp27B1	25-hidroxivitamina D3 1-α-hidroxilasa
DAD	dominio activador de desacetilasas
DAX-1	reversión de sexo sensible a dosis
DBD	dominio de unión al DNA
DR	repeticiones directas separadas por un número variable de nucleótidos
DRIP	proteínas que interaccionan con el VDR
ER	receptor de estrógenos
ERR	receptor relacionado con estrógenos
Ets	factores de transcripción "E-Twenty-Six"
Fos	oncogén del virus Finkel-Biskis-Jinkins (FBJ) de osteosarcoma
FTZ-1/SF-1	Fushi-Tarazu/factor esteroidogénico 1
FXR	receptor X de farnesoides
GCNF	factor nuclear de la línea germinal
GFT	factor de transcripción general o basal
GR	receptor de glucocorticoides
H	hélice α

HAT	acetiltransferasa de histonas
HDAC	desacetilasa de histonas
HMT	metiltransferasa de histonas
HNF-4	factor nuclear de hepatocitos 4
HRE	elemento de respuesta a hormona
Hsp90	proteína de choque térmico 90
IP	palíndromo invertido
ISWI	imitación de SWI
LBD	dominio de unión al ligando
LCA	ácido litocólico
LCoR	correpresor de receptores nucleares dependiente de ligando
LXR	receptor X del hígado
MR	receptor de mineralocorticoides
NCoA62	coactivador nuclear de 62 kDa
NCoR	correpresor nuclear
NGF-1	clon B inducido por el factor de crecimiento nervioso NGF
NR	receptor nuclear
N-terminal	amino-terminal
NURD	remodelación de nucleosomas y desacetilación
p300	proteína asociada a la proteína de adenovirus E1A
PAH	hélices anfipáticas por pares
Pal	palíndromos
PARP1	poli (ADP-ribosa)-polimerasa 1
PAS	"Per-Arnt-Sim" o "Period/Aryl hydrocarbon receptor/Single minded homology"
PCAF	factor asociado a p300/CBP
PGC-1	coactivador 1 de PPARγ
Pit-1	factor específico de hipófisis 1
POMC	pro-opiomelanocortina
PPAR	receptor de los activadores de la proliferación de los peroxisomas
PR	receptor de progesterona
PRAME	antígeno preferentemente expresado en melanoma
PRMT	proteína arginina metiltransferasa
PXR	receptor X de pregnanos
RAR	receptor de ácido retinoico
RAREβ	elemento de respuesta al ácido retinoico presente en el promotor RARβ2
RD	dominio represor
REA	represor de la actividad del receptor de estrógenos
RevErb	Erb reverso
RID	dominio de interacción con los receptores nucleares
RIP140	proteína de 140 kDa que interacciona con el receptor

ROR	receptores huérfanos relacionados con los de retinoides
RXR	receptor X de retinoides
SHP	pequeño compañero heterodimérico
Sin3	"switch independent-3"
SMRT	mediador del silenciamiento por RAR y TR
SRA	RNA activador de receptores esteroideos
SRB	supresor de la RNA-polimerasa B
SRC-1	coactivador de receptores esteroideos 1
SREBP	proteína de unión a elementos reguladores de esteroles
SUMO	pequeño modificador relacionado con la ubiquitina
SUN-CoR	pequeño correpresor nuclear ubicuo
SWI-SNF	complejo "mating-type switch/sucrose nonfermenting"
T3	triyodotironina
T4	tetrayodotironina o tiroxina
TAF	factores asociados a TBP
TBG	globulina ligadora de tiroxina
TBL1	proteína 1 de tipo transducina beta
TBP	proteína de unión a la caja TATA
TEF	factor del "enhancer" transcripcional
TFII	factor de transcripción II
TIF-2	factor transcripcional intermediario 2
TLS	translocado en liposarcoma
TR	receptor de hormonas tiroideas
TR2	receptor de testículos
TRAP	proteínas asociadas al TR
TRE	elemento de respuesta a hormonas tiroideas
Trip-1	proteína 1 que interacciona con TR
TSC-2	esclerosis tuberosa 2
TSH	hormona tirotropa
Ubc9	conjugante 9 de ubiquitina
VDR	receptor de vitamina D
VDRE	elemento de respuesta a la vitamina D
VDRM	modulador del VDR
v-erbA	oncogén del virus de la eritroblastosis aviar
VitD	1α,25 dihidroxivitamina D3
VP16	proteína viral 16 del virus del *Herpex simplex*
WINAC	complejo de ensamblaje de nucleosomas que incluye WSTF
WSTF	factor de transcripción del síndrome de Williams

Introducción

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1. La Superfamilia de los Receptores Nucleares

Las hormonas tiroideas, los retinoides y la vitamina D son compuestos lipofílicos de bajo peso molecular que controlan una gran variedad de procesos biológicos en los metazoos como desarrollo, homeostasis, diferenciación y morfogénesis. Concretamente las hormonas tiroideas, triyodotironina (T3) y tetrayodotironina o tiroxina (T4), desempeñan papeles fundamentales regulando el consumo de oxígeno y la tasa metabólica, el desarrollo y crecimiento del sistema nervioso y del hueso y la función cardiaca (143, 219). Los derivados de la vitamina A conocidos como retinoides, por su parte, tienen efectos sobre la morfogénesis en el embrión, la diferenciación del sistema nervioso y de los epitelios, además de regular la inflamación y la proliferación celular (33, 166). Las acciones más destacadas del calcitriol (1,25-OH_2-vitamina D3, metabolito activo de la vitamina D) incluyen el mantenimiento de la homeostasis del calcio y del fósforo, la modulación del sistema inmunitario y la diferenciación de células cancerosas (42, 41).

A diferencia de las hormonas de naturaleza polipeptídica, que ejercen sus acciones a través de receptores de membrana acoplados a cascadas de señalización celular que conducen a la regulación de la expresión génica; la mayoría de los efectos biológicos de las hormonas tiroideas, los retinoides, ácido-todo-*trans*-retinoico (atRA) y ácido 9-*cis*-retinoico (9-*cis*RA), o el calcitriol (VitD), están mediados fundamentalmente a través de la unión a receptores que se encuentran en el núcleo celular. Se trata de un grupo de factores de transcripción dependientes de ligando que se engloban en la conocida como "superfamilia de los receptores nucleares" (128). Esta superfamilia comprende no sólo los receptores de las hormonas anteriormente mencionadas, sino también los receptores para hormonas esteroideas, diversos productos del metabolismo lipídico, ácidos biliares, e incluso receptores para los que no existe ningún ligando conocido, los llamados "receptores huérfanos" (162). Los receptores nucleares (NRs) son uno de los grupos más extensos de factores de transcripción, identificándose más de 40 genes que codifican para este tipo de proteínas en mamíferos. Desde el punto de vista evolutivo, se cree que todos ellos derivan de un único gen ancestral común, siendo la unión de ligando una capacidad adquirida durante el transcurso de la evolución (54, 111).

1.1. Clasificación de los Receptores Nucleares

El análisis evolutivo ha llevado a la división de los NRs en 6 subfamilias (71). La primera gran subfamilia comprende a los receptores de hormonas tiroideas (TRs), los de ácido retinoico (RARs), los de vitamina D (VDRs), los receptores de los activadores de la proliferación de los peroxisomas (PPARs), así como varios receptores huérfanos algunos de los cuales han sido recientemente "adoptados" como el receptor X de farnesoides (FXR), el receptor constitutivo de androstanos (CAR), el receptor X del hígado (LXR), el receptor X de pregnanos (PXR) y los receptores huérfanos relacionados con los de

retinoides (RORs). Una segunda subfamilia incluiría a los receptores X de retinoides (RXRs), los estimuladores "upstream" de la ovoalbúmina de pollo (COUPs), los receptores de testículos (TR2) y el factor nuclear de hepatocitos 4 (HNF-4), entre otros. La tercera subfamilia la integran los receptores de hormonas esteroideas; como los de estrógenos (ERs) y receptores relacionados con estrógenos (ERRs), andrógenos (ARs), progesterona (PRs), glucocorticoides (GRs), y mineralocorticoides (MRs). La cuarta, quinta y sexta subfamilias comprenden a los receptores huérfanos NGF-1, FTZ-1/SF-1 y GCNF (68). A veces se distingue una subfamilia o clase 0, formada por SHP y DAX-1 (5).

A su vez, dentro de cada subfamilia pueden establecerse también subtipos correspondientes a las distintas isoformas de cada receptor (5). Por ejemplo, los TRs, están codificados por dos genes distintos, TRα y TRβ. Cada uno de ellos da lugar a dos isoformas, que surgen por procesamiento alternativo, resultando en TRα1 TRα2 TRβ1 y TRβ2. El caso de los retinoides es aún más complejo. Tanto para RAR como para RXR, existen 3 subtipos α, β y γ; codificados por diferentes genes que mediante diferencias de procesamiento o uso de promotores alternativos generan 6 isoformas de RXR y hasta 18 de RAR. El caso de VDR es bien distinto, ya que sólo ha sido descrito un único gen que lo codifique. Las distintas variantes de NRs se expresan diferencialmente según el tipo celular y el momento del desarrollo, pudiendo tener funciones diferentes y complementarias. Se dan casos de cierta redundancia en el que la ausencia de alguna isoforma en un tejido es suplida por otra, como revela el estudio mediante ratones "knock out" de varios subtipos de NRs.

2. Mecanismo de Acción

2.1. Fisiología de los ligandos de los receptores nucleares

Dado que los ligandos de los NRs tienen diversas procedencias y naturalezas químicas, el mecanismo y la forma en la cual llegan hasta las secuencias de unión en los genes que regulan es variado.

Existen ligandos de carácter endocrino que se sintetizan en otro tejido distinto al diana, y llegan hasta su destino viajando por el torrente sanguíneo unidos a proteínas transportadoras. Es el caso de hormonas esteroideas como los corticoides o las hormonas sexuales y de las hormonas tiroideas. Concretamente las hormonas tiroideas, T3 y T4, se sintetizan en la glándula tiroides a partir de la yodación de una proteína altamente especializada y rica en residuos tirosínicos, la tiroglobulina. La hidrólisis de esta proteína, da lugar a la síntesis y liberación a la sangre de T4 y T3 donde son transportadas unidas a diferentes proteínas como TBG y albúmina. Aunque la T4 es la hormona segregada mayoritariamente por el tiroides, funciona como una prohormona, siendo su desyodación en los distintos tejidos la mayor fuente de producción de T3, la forma biológicamente más activa y de mayor afinidad por su receptor nuclear TR.

Otro tipo de ligandos se encuentran en forma de precursores inactivos que se convierten en su forma activa al ser metabolizados en la célula diana, como los retinoides; o bien en otros órganos o tejidos, como la vitamina D. Los retinoides sólo pueden ser incorporados por los animales a través de la

dieta. Sus fuentes principales son los carotenoides procedentes de las plantas y los ésteres de retinilo de cadena larga presentes en los tejidos animales. La modificación enzimática de estos compuestos en el intestino da lugar a la formación del retinol, que se transporta por la sangre acomplejado con proteínas específicas, y es esterificado o incorporado a partículas lipoproteicas. Finalmente en las células diana es convertido a atRA y 9-*cis*RA, que se unirán a sus receptores en el núcleo celular, RAR y RXR. Mientras que el RAR une ambos compuestos con alta afinidad, el RXR sólo une 9-*cis*RA. La vitamina D, por su parte, puede sintetizarse en la piel en respuesta a la radiación UV a partir del 7-deshidrocolesterol, o bien incorporarse a través de la dieta. La forma así generada se conoce como colecalciferol y su posteriores deshidroxilaciones sucesivas en el hígado y riñón darán lugar al calcitriol o 1α,25-dihidroxivitamina D3, la forma activa responsable de la activación del receptor, VDR. El calcitriol es inactivado para su posterior excreción a través de una hidroxilación en la posición 24 por intervención de la enzima 1α,25-dihidroxivitamina D3 24-hidroxilasa (Cyp24). Esta enzima es especialmente importante por presentar una de las mayores activaciones conocidas en respuesta al ligando de un NR, como veremos más adelante.

Por último, algunos ligandos de receptores nucleares pueden ser generados dentro de la célula diana como parte de su metabolismo, es el caso de algunas prostaglandinas y ácidos grasos que se unen a receptores como PPAR o los derivados del colesterol que se unen al LXR.

2.2. Localización subcelular

Otro aspecto fundamental para entender el mecanismo de acción de estos receptores es la localización subcelular de los mismos (75). Tradicionalmente se consideraba que los receptores nucleares vacíos eran proteínas citosólicas. El receptor de glucocorticoides (GR) en ausencia de ligando se encuentra asociado a otras proteínas, entre ellas la proteína de choque térmico Hsp90, que lo retienen en el citosol. Tras la unión del ligando el receptor sufre un cambio conformacional por el cual se disocia del complejo citosólico y es transportado al núcleo. Datos más recientes indican la presencia de ER, PR y MR en el núcleo incluso en ausencia de ligando. Se piensa que esta presencia podría estar implicada en mantener el gen diana listo para la activación transcripcional cuando la estimulación por el ligando se produjese (129). No se puede excluir que los receptores de esteroides sean de localización tanto citoplásmica como nuclear con un equilibrio nucleo-citoplásmico desplazado hacia el citoplasma en el caso de GR y hacia la fracción nuclear en el caso de los esteroides gonadales. En el caso de VDR también se postula una distribución equilibrada entre los dos compartimentos celulares. Parece bastante establecido que los receptores de hormonas tiroideas son de localización nuclear en ausencia de hormona, y lo mismo ocurre con los receptores de ácido retinoico y algunos receptores "huérfanos". En cualquier caso, tras su llegada al núcleo los complejos hormona-receptor se unirían a secuencias específicas de DNA los denominados "elementos de respuesta hormonal" o HREs y como consecuencia se produciría un cambio en la velocidad de la transcripción de los genes que contienen dichos elementos.

2.3. Elementos de respuesta

Los NRs regulan la expresión génica por medio de la unión a secuencias específicas de DNA denominadas **elementos de respuesta a hormona (HREs).** Existen varios tipos de HREs a los cuales se unen los receptores en forma de monómeros o dímeros (69). En el caso de los receptores no esteroideos estos elementos de respuesta están formados por un motivo de reconocimiento derivado de la secuencia AGGTCA (hemisitio), pudiendo ser un motivo único precedido de una zona rica en A y T para los monómeros, o duplicado (dos hemisitios) para los dímeros. Estos hemisitios pueden configurarse como palíndromos (Pal), palíndromos invertidos (IPs) o repeticiones directas (DRs) separadas por un número variable de nucleótidos. Mientras que los receptores de hormonas no esteroideas tienen elementos de respuesta con distintas configuraciones, los receptores de esteroides únicamente reconocen elementos de tipo palindrómico. En el caso de las repeticiones directas (DRs), la longitud de la región espaciadora es un importante determinante de la especificidad de la respuesta hormonal. Así, motivos separados por 3, 4 y 5 nucleótidos (DR3, DR4 y DR5) median la unión de VDR, TR y RAR, respectivamente (133, 198). En cambio, un DR1 actúa como elemento de unión tanto para RXR, como para PPAR. El tipo de elemento de respuesta también puede determinar la orientación con la que se unen los heterodímeros del RXR. Por ejemplo, el RAR ocuparía la posición 3' de la pareja en un elemento de tipo DR5, mientras que cuando el heterodímero RAR/RXR se encuentra unido a un DR2, es el RXR el que ocuparía esa posición (127).

2.4. Monómeros y dímeros

Los NRs pueden unirse a sus HREs en el DNA como **monómeros, homodímeros o heterodímeros** para ejercer sus funciones como factores de transcripción. Algunos receptores huérfanos como SF-1 y ROR, se unen al DNA como monómeros (68). Sin embargo, la mayoría de receptores nucleares actúan en forma de dímeros, bien sean homodímeros, como los receptores de hormonas esteroideas, o heterodímeros con el RXR como pareja, como es el caso de los receptores de hormonas no esteroideas TR, RAR, VDR, PPAR y otros (25, 101, 102, 112). En el caso del RXR, este puede unirse a sus elementos de respuesta DR1 como homodímero (229), o bien como heterodímero con los receptores anteriormente mencionados.

2.5. Heterodímeros del RXR

Como hemos visto, gran cantidad de NRs regulan la expresión génica uniéndose al DNA en forma de heterodímeros con el RXR. Se han descrito 3 tipos de heterodímeros de NRs, permisivos, no permisivos y condicionales (179). Esta distinción se hace en base a la capacidad que tenga el RXR dentro de estos heterodímeros de unir su propio ligando, el 9-*cis*RA y de activar la transcripción. En teoría, se

predicen 4 estados diferentes de ocupación por el ligando de estos heterodímeros: RXR ocupado, su pareja ocupada, ambos receptores ocupados y ambos receptores vacíos.

Los heterodímeros **permisivos** como PPAR/RXR, FXR/RXR, LXR/RXR o NGFI-B/RXR, podrían activarse en respuesta a los ligandos de cada uno de los miembros de la pareja indistintamente, y se verían sinérgicamente activados en presencia de los dos ligandos simultáneamente.

Por el contrario, en los heterodímeros **no permisivos**, la actividad transcripcional del RXR se encontraría suprimida debido a que la propia heterodimerización evitaría la unión del 9-*cis*RA a su receptor (62). Así pues, se habla de que el RXR actuaría como un "compañero silencioso" en este tipo de complejos. Tradicionalmente han sido definidos como no permisivos los heterodímeros formados por VDR y TR. El significado biológico para esta subordinación o silenciamiento del RXR probablemente pudiese ser evitar el solapamiento entre las vías de señalización de estos receptores.

Recientemente se ha postulado para el heterodímero RAR/RXR (antes considerado como no permisivo), un nuevo modelo de heterodímero denominado **condicional**, en el cual sólo se obtendría una respuesta transcripcional completa al ligando de RXR en presencia del agonista de su compañero (66, 179).

En el caso del heterodímero LXR/RXR, la estimulación por 9-*cis*RA requiere el dominio AF-2 de LXR, pero no del de RXR, lo que demuestra que la unión del agonista de RXR provoca un cambio conformacional en el LXR que conduce a la activación transcripcional. Este fenómeno ha sido definido como el efecto del "ligando fantasma" (209). Además, se ha identificado un retinoide sintético específico para RXR que también se comporta como un "ligando fantasma" mimetizando la acción de los agonistas de RAR, sin ocupar su cavidad de unión a ligando (172).

3. Estructura de los Receptores Nucleares

Los NRs presentan una estructura modular en la que las distintas regiones se corresponden con dominios funcionales autónomos que pueden ser intercambiados entre receptores relacionados sin que se produzca una pérdida de función. Un receptor nuclear típico se compone de seis dominios que se nombran con letras de la A a la F desde el extremo N-terminal al C-terminal (Figura 1). Así pues, se distingue una región variable A/B, un dominio conservado C de unión al DNA o DBD, una región bisagra D, un dominio E de unión al ligando (LBD) y en algunos casos una región C-terminal o dominio F. Los NRs presentan además dos regiones de las que depende la activación transcripcional, son las llamadas funciones de activación 1 y 2, AF-1 y AF-2 respectivamente. La primera se encuentra localizada en la región hipervariable A/B y contribuye a la activación constitutiva del receptor independiente de ligando. La región AF-2 se encuentra en el extremo carboxilo del LBD y media la transactivación en respuesta al ligando.

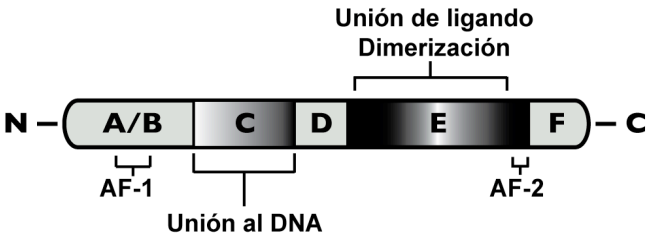

Figura 1. Estructura de un receptor nuclear. Los receptores nucleares presentan varios dominios funcionales. La región A/B incluye el dominio de transactivación independiente de ligando AF-1. En la región C o DBD reside la unión al DNA. La región D o bisagra conecta el DBD con la región E o dominio de unión a ligando (LBD) que contiene la función de activación transcripcional dependiente de ligando AF-2.

3.1. La región A/B

Este dominio de naturaleza moduladora es el más variable de los seis que componen los NRs tanto en secuencia como en tamaño. En esta región difieren la mayoría de las isoformas de receptores que se originan por procesamiento diferencial o por el uso de promotores alternativos. En la mayoría de los casos contiene el dominio AF-1 que media la transactivación independiente del ligando. Esta región AF-1 es especialmente importante en el caso de algunos receptores como AR o PPARα. Por otro lado, en este dominio A/B se localizan residuos diana de la fosforilación por quinasas que se activan por distintas rutas de señalización celular pudiendo influir en la actividad transcripcional de los receptores (164). Es el caso de la fosforilación por la MAPK de los receptores ER y RXR (97, 183).

3.2. El dominio de unión al DNA

También llamado región C, este dominio es el más conservado y por tanto el que muestra mayor homología entre los distintos miembros de la superfamilia. Contiene nueve cisteínas así como otros residuos muy conservados fundamentales para el reconocimiento de la secuencia de DNA a la cual se han de unir. El DBD está compuesto por dos "dedos de zinc", donde las cisteínas se coordinan de cuatro en cuatro en torno a un átomo de zinc, y una extensión C-terminal donde se encuentran las cajas T (tandem) y A (adenina). Todo ello forma una estructura compacta y determinada que toma la forma de dos hélices α. La primera de las hélices, denominada de reconocimiento, se une al surco mayor del DNA en el elemento de respuesta y la segunda hélice se dispone perpendicularmente a la anterior, tal y como han demostrado los estudios cristalográficos de la región (122, 173). Los dedos de zinc contienen dos importantes motivos aminoacídicos llamados cajas P (proximal) y D (distal). La caja P se encuentra en la base del primer dedo de zinc y contiene los residuos encargados de discriminar el motivo específico al que se une el receptor. La caja D se encuentra en el segundo dedo de zinc y está encargada de la dimerización en los receptores que así lo requieran y en el reconocimiento del espaciamiento característico de cada tipo de HRE. En cuanto a la extensión C-terminal, parece que las cajas T y A estarían implicadas en reforzar el afianzamiento sobre el DNA de los receptores que se unen como monómeros y que por tanto carecen de la estabilidad proporcionada por la dimerización (161).

3.3. El dominio D

Esta región desempeña funciones de bisagra entre el dominio de unión a ligando (LBD) y el DBD permitiendo la rotación de este último. No es un dominio muy conservado entre los distintos receptores y en bastantes casos posee señales de localización nuclear y residuos importantes para la interacción con correpresores, como demuestra el análisis mutacional de los mismos (37, 86). La región D está íntimamente asociada al LBD en presencia del ligando o de correpresores. Este rasgo permite un efecto estabilizador de la estructura general del receptor (151).

3.4. El dominio de unión al ligando

La región E es la segunda más conservada de los NRs. Además de ser responsable de la selectividad de unión al ligando, este dominio multifuncional media la homo y heterodimerización, la interacción con proteínas de choque térmico, coactivadores, correpresores y mediadores, y por tanto la activación, y en algunos casos la represión, transcripcional dependiente de ligando. El LBD contiene dos regiones bien conservadas, el **motivo Ti** y el motivo C-terminal **AF-2**, responsable de la activación dependiente de ligando. La estructura cristalográfica de los LBDs de varios receptores revela una estructura general bien conservada formada por 12 hélices α (Hs) numeradas desde H1 hasta la H12 con una lámina β dispuesta entre las hélices H5 y H6. El LBD se pliega en una estructura en tres capas antiparalelas. La capa central se empaqueta entre las otras dos creando una cavidad donde se acomoda el ligando.

3.4.1. La cavidad de unión al ligando

Una cavidad constituida por aminoácidos hidrofóbicos acomoda al ligando en el interior de la estructura del LBD. El tamaño de esta cavidad es variable entre los distintos receptores, siendo pequeño en los receptores clásicos de hormonas endocrinas que presentan alta afinidad por el ligando y muy grande en los receptores con capacidad de unir distintos tipos de ligandos pero con baja afinidad. En algunos receptores huérfanos, la cavidad de unión de ligando se encuentra casi colapsada por residuos con voluminosas cadenas laterales, prácticamente imposibilitando la unión de un hipotético ligando.

3.4.2. La función de dimerización

Los NRs poseen dos regiones principales de dimerización. La primera reside como ya describimos en el DBD y la segunda, de mayor importancia, está mediada por las interfases formadas por las hélices 9 y 10 así como el giro que va desde la H7 a la H8 de cada receptor implicado. La interfase de dimerización consta de nueve repeticiones de motivos en las que el séptimo aminoácido está muy conservado, conocidas como "heptadas de dimerización" (61). En particular, la novena heptada parece ser crítica para

la dimerización de varios receptores; de hecho, forma parte de la H10, el mayor componente estructural de la superficie de dimerización (6, 57).

Dado que los NRs contienen 2 regiones de dimerización independientes, una en el DBD y otra en el LBD, y que parecen funcionar de modo secuencial, se ha propuesto la llamada "**hipótesis en 2 etapas**" para la formación de heterodímeros. En una primera etapa RXR podría formar en solución heterodímeros con sus compañeros a través de sus LBDs. En una segunda etapa los DBDs, ya muy próximos, podrían unirse a sus sitios de unión en el DNA (127). La diversidad de tipos de elementos a los que los heterodímeros pueden unirse implica que los DBDs deben ser capaces de rotar al menos 180º con respecto a la superficie de dimerización en el LBD, lo que se logra gracias a la flexibilidad que proporciona el dominio D a estos receptores.

3.4.3. El dominio AF-2

Esta región responsable de la activación dependiente de ligando se encuentra en el extremo C-terminal del LBD correspondiéndose prácticamente con la H12 completa. Este dominio presenta gran homología entre los miembros de la superfamilia y posee un motivo consenso ϕϕXEϕϕ, donde ϕ es cualquier aminoácido hidrofóbico (206, 225). Aunque el núcleo de la función de activación dependiente de ligando reside en la H12, existen otros elementos fundamentales en cuanto a los cambios que acontecen en el LBD tras la unión del ligando. Es el caso del **motivo Ti**, secuencia de unos 20 aminoácidos que comprende el extremo C-terminal de la H3, el giro que va de la H3 a la H4 y el extremo N-terminal de la H4. Se asume que esta región conservada tiene función estructural, asegurando la estabilidad del LBD. La mutación de este motivo no afecta ni a la unión del ligando ni a la dimerización, pero impide la activación transcripcional dependiente de ligando. Concretamente, una lisina situada en el extremo C-terminal de la H3 juega un papel crucial en la transactivación de ciertos receptores (214).

Se observan notables diferencias al comparar la estructura de NRs vacíos y en presencia de ligando. Las estructuras ligadas son más compactas que las formas vacías, tal y como demuestra la mayor movilidad electroforética y resistencia a la proteolisis de las primeras. Todo esto sugiere que la unión del ligando desencadena un cambio conformacional en estos receptores. Los estudios cristalográficos han permitido demostrar que la diferencia más notable entre las dos conformaciones reside en la disposición de la H12 (161). La integridad de esta hélice anfipática es requisito indispensable para la activación dependiente de ligando (12, 45, 52). La estructura del LBD de RXR vacío, muestra cómo la H12 se proyecta hacia el exterior formando un ángulo de 45º respecto al cuerpo central del LBD. Por el contrario, en los receptores que han unido ligando, la H12 se empaqueta estrechamente contra las hélices 3 y 4, estableciendo contactos directos con el ligando y cerrando, por tanto, la cavidad que lo contiene. Esta disposición genera un surco hidrofóbico que permite una superficie adecuada para el reclutamiento de coactivadores requeridos para la activación de la transcripción (131). En el caso del receptor de estrógenos unido a un ligando antagonista, la posición de la H12 es diferente, solapando con el surco hidrofóbico e interfiriendo con la unión de los coactivadores (24, 178). Además de los cambios en la

posición de la H12, la unión del ligando desencadena cambios importantes en otras regiones del LBD. Concretamente, la H11 se dispone a continuación de la H10 y el desplazamiento asociado de la H12 permite que el giro **Ω** (entre H2 y H3) se coloque debajo de la H6 arrastrando la parte N-terminal de la H3 (Figura 2).

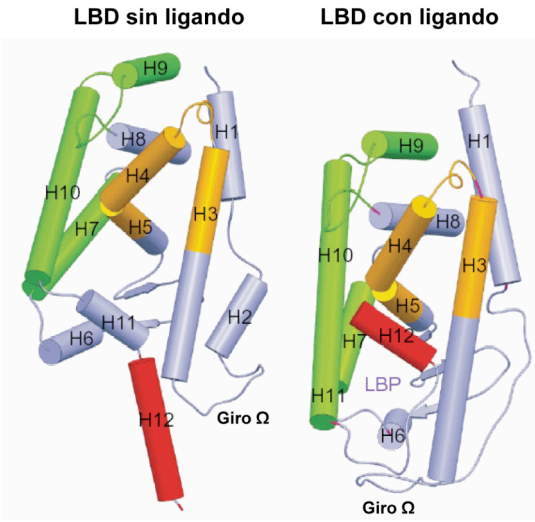

Figura 2. Cambio conformacional tras la unión del ligando. Se representan la estructuras cristalográficas de los LBDs de RXRα (izquierda) sin ligando y de RARγ (derecha) unido a su agonista. Los cilindros representan las 12 hélices α denominadas desde H1 hasta H12. Las flechas representan segmentos de láminas β. Se destaca la distinta posición en ambas estructuras de la H12 (color rojo), la cual contiene el núcleo de la función de activación transcripcional dependiente de ligando AF-2. LBP: cavidad de unión del ligando.

4. Regulación de la expresión génica por los receptores nucleares

4.1. Los receptores nucleares y la maquinaria de transcripción basal

La ocupación de los NRs por el ligando conduce a la activación transcripcional de los genes cuyos promotores contienen los HREs. Los promotores transcritos por la RNA-polimerasa II son reconocidos por dos tipos de factores de transcripción, los factores de transcripción generales o basales (GFTs), que interaccionan con los elementos centrales del promotor, y los factores de transcripción específicos de secuencia, entre los que se encuentran los NRs, que se suelen unirse a secuencias más distales del inicio de la transcripción. En el caso de los genes con caja TATA, el inicio de la transcripción depende de la unión de diferentes factores de transcripción generales al promotor. El complejo de iniciación transcripcional consta de la RNA-polimerasa II (que se compone de al menos 12 subunidades) y de los factores de transcripción generales TFIID, TFIIB, TFIIA, TFIIF, TFIIE y TFIIH (210). Los GTFs por si solos pueden determinar la especificidad de la RNA-polimerasa II y dirigir bajos niveles de transcripción, así como responder a factores de transcripción dependientes de secuencia o activadores.

Se pensaba que el complejo de preiniciación de la transcripción se ensamblaba sobre las secuencias promotoras de los genes de una forma ordenada y sucesiva (26), ocurriendo primero la unión de TFIID, seguida de la unión de TFIIB, la RNA-polimerasa II y otros factores. Actualmente se sabe que en la célula existen complejos preformados que incluyen la RNA-polimerasa II, TFIIB, TFIIH, TFIIF y SRBs entre otras proteínas, denominados "holoenzima", que pueden ser reclutados directamente al promotor por los

factores de transcripción. En este caso, el ensamblaje del complejo de iniciación de la transcripción quedaría reducido a la unión de TFIID, TFIIA, el holoenzima y TFIIE. El factor TFIID está compuesto de la proteína TBP, que se une a la caja TATA y de los factores asociados a la TBP, denominados $TAF_{II}s$.

Los efectos de los factores de transcripción sobre la expresión génica parecen deberse en parte a la influencia sobre la tasa de ensamblaje de los complejos de maquinaria transcripcional basal al promotor. Los NRs pueden actuar sobre la velocidad de la transcripción génica, a través de la interacción con diferentes componentes del complejo de preiniciación. Estas interacciones pueden ser directas, y de hecho se ha demostrado que los NRs contactan directamente con los GTFs incluyendo TBP, ciertos TAFs, TFIIB y TFIIH. Por ejemplo, se ha descrito interacción de TBP con los receptores TR y RXR (170), potenciando la transactivación dependiente de ligando causada por dichos receptores. También se han descrito interacciones del factor TFIIB con TR y VDR con distintos efectos transcripcionales (8, 20, 168).

Sin embargo, la modulación del ensamblaje de los complejos de preiniciación de la transcripción al promotor por los NRs implica no sólo las acciones directas sobre la maquinaria basal, sino acciones indirectas mediadas a través de otras proteínas, los **correguladores**, fundamentalmente coactivadores y correpresores.

4.2. Activación transcripcional dependiente de ligando. Coactivadores

Los coactivadores son moléculas puente que median la interacción de los factores de transcripción con la maquinaria de transcripción basal. El empaquetamiento de la cromatina en nucleosomas representa un impedimento para la transcripción dado el grado de compactación. Existen 2 mecanismos principales que causan la descompactación de la cromatina y alivian el bloqueo transcripcional derivado de la estructura en nucleosomas; por una parte, las histonas pueden ser modificadas post-traduccionalmente, lo cual desestabiliza la cromatina (212), y por otro lado, los nucleosomas pueden ser desestructurados por la acción de maquinaria dependiente de ATP (201). Por ello, no sorprende que muchos coactivadores sean factores remodeladores de cromatina dependientes de ATP o proteínas con actividad acetilasa, metilasa o ubiquitina ligasa, o que bien interaccionen directamente con la maquinaria basal y ayuden al reclutamiento de la polimerasa al promotor.

4.2.1. Acetilasas de histonas

Las histonas pueden sufrir una gran variedad de modificaciones post-traduccionales que incluyen acetilación, metilación, fosforilación, ubiquitinación, sumoilación y ADP-ribosilación. Normalmente esas modificaciones ocurren en los extremos amino o carboxilo de las colas de las histonas, que juegan un importante papel en el control del empaquetamiento de los nucleosomas en estructuras de un orden mayor. Algunas de estas modificaciones de las histonas provocan una descompactación de la cromatina y permiten un mayor y mejor acceso de los factores de transcripción al promotor, además de poder crear nuevos sitios de reconocimiento para otra serie de reguladores positivos. Por ejemplo, los factores con

bromodominios reconocen lisinas acetiladas, mientras que las metiladas son reconocidas por factores con cromodominios. Todo esto ha llevado a la elaboración de la **hipótesis del "código de histonas"** (91), por la cual, combinaciones específicas de modificaciones de histonas pueden determinar la activación y la represión transcripcional.

4.2.2. Coactivadores p160

Las proteínas de la familia de coactivadores SRC/p160 se encuentran entre los primeros factores identificados que interaccionan con los NRs en presencia de ligando. Se caracterizan por presentar un peso molecular de aproximadamente 160 kDa e integran esta familia 3 miembros relacionados entre si: SRC-1/NCoA-1 (142), TIF-2/SCR-2/GRIP-1/NCoA-2 (203) y ACTR/p/CIP/AIB1/TRAM1/RAC3 (4, 36). Estas proteínas actúan como coactivadores primarios, interaccionando con distintos NRs de manera dependiente de agonista, siendo indispensable para la interacción el dominio AF-2 de los receptores. Por otro lado, estos coactivadores sirven también como plataformas de anclaje de otros coactivadores secundarios.

Los miembros de la familia p160 de coactivadores presentan un patrón estructural común y bastante conservado (Figura 3). Poseen un dominio de interacción con los receptores nucleares (RID) en su región central. El extremo N-terminal, el más conservado de la estructura de estos coactivadores, contiene la señal de localización nuclear, un dominio de tipo bHLH y un dominio PAS. Estos dominios median la interacción con el coactivador secundario CoCoA (98), con BAF57 (14), componente del complejo SWI-SNF (que explicaremos más adelante) y con factores de transcripción de la familia TEF (15). Los coactivadores p160 poseen también una región rica en serina y treonina y otra región C-terminal rica en glutamina, también muy bien conservadas. Hacia el extremo C-terminal se encuentran dos dominios de activación, AD1 y AD2 implicados en la interacción con acetiltransferasas como CBP/p300 y PCAF y con metiltransferasas como CARM1 (35), respectivamente. Por otro lado, tanto SRC-1 como ACTR poseen actividad HAT (acetiltransferasa de histonas) intrínseca residente en su región C-terminal, aunque no se ha descrito dicha actividad en el caso de TIF-2 (36, 184).

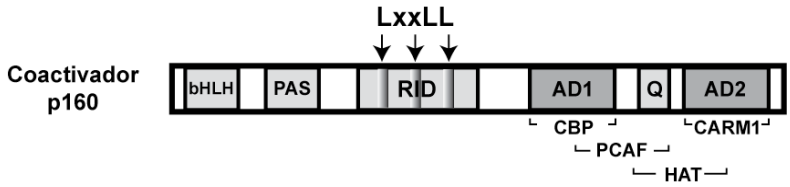

Figura 3. Estructura de un coactivador p160. Estas proteínas contienen un dominio bHLH y uno de homología PAS en su extremo N-terminal. El dominio de interacción con los NRs contiene 3 copias del motivo LxxLL indicadas por barras verticales. En el extremo C-terminal se encuentran los 2 dominios activadores AD1 y AD2 y una región rica en glutamina (Q). Los corchetes indican la región donde reside la actividad HAT así como las zonas de interacción con las acetilasas CBP/p300 y P/CAF y la metiltransferasa CARM1.

El RID de los coactivadores p160 contiene 3 copias del motivo altamente conservado LxxLL, donde L es leucina y x un aminoácido cualquiera. Estos motivos son tanto necesarios como suficientes para mediar la interacción de los NRs con los coactivadores en presencia de ligando (49, 81, 196). Los motivos LxxLL se configuran como hélices α anfipáticas, en las que las leucinas forman una superficie hidrofóbica a un lado de la hélice. Un residuo conservado de ácido glutámico presente en la H12 de los receptores, así como un residuo de lisina presente en la H3 también conservado en toda la superfamilia, hacen contactos directos con las leucinas 1 y 5 del motivo LxxLL de los coactivadores. Se forma así una estructura que orienta y posiciona al coactivador por medio de una zona de carga en el surco hidrofóbico formado en el receptor tras la unión del ligando (46). Una única molécula de coactivador puede interaccionar con los dominios AF-2 de los dos miembros de la pareja del homo o heterodímero a través de dos de sus motivos LxxLL (139).

4.2.3. CBP/p300

CBP, la proteína que se une a CREB y p300 (proteína asociada a la proteína de adenovirus E1A), debido a su alta homología son denominadas ambas por simplicidad CBP/p300. Contienen varios dominios funcionales, como el dominio de interacción con CREB denominado KIX, tres regiones con dedos de zinc que unen factores como la acetilasa PCAF, y un dominio con actividad intrínseca HAT (10, 141). CBP/p300 interacciona con los NRs por su extremo N-terminal (94) que contiene un motivo del tipo LxxLL. Esta interacción es dependiente de ligando y de la región AF-2 del receptor, por lo que CBP/p300 parece ser uno de los coactivadores fundamentales para la acción de los NRs (32). Por otro lado CBP/p300 también es capaz de interaccionar con los coactivadores p160 a través su región C-terminal (196, 202), lo que proporciona a los NRs dos maneras distintas de interaccionar con CBP/p300; o bien a través de interacción directa con el extremo N-terminal de CBP/p300, o bien a través de la interacción con los coactivadores p160. Puesto que CBP/p300 utiliza distintas regiones para la interacción con los NRs y con los coactivadores, es posible la formación de complejos ternarios.

CBP/p300 funciona como coactivador para múltiples factores de transcripción como AP-1, NF-κB, Pit-1 y Ets, además de los NRs, por lo que se le atribuye un papel integrador entre distintas vías de señalización celular. Atendiendo a esta función integradora, en un principio se atribuyó a la competición por cantidades limitantes de CBP/p300 el antagonismo recíproco de la acción de los complejos AP-1 (Jun/Fos) y diversos miembros de los NRs como GR o RAR (94).

Por otro lado se han detectado interacciones de CBP/p300 con factores como TBP o TFIIB, lo que podría servir para conectar a los NRs con la maquinaria de transcripción basal (107).

4.2.4. La acetiltransferasa de histonas PCAF

El factor asociado a p300/CBP (PCAF) fue la primera proteína con actividad HAT identificada en mamíferos. Interacciona directa e independientemente con los NRs y con coactivadores p160 y

CBP/p300 a través de regiones distintas, actuando entonces como un coactivador (19, 104). Por tanto, PCAF ejerce un papel dual en la activación de la transcripción por los NRs, por un lado como acetiltransferasa tiene la capacidad para modificar la cromatina hacia la reversión de la represión génica y por el otro, su capacidad de interacción con los p160 y con CBP/p300 le permiten reclutar activadores adicionales con actividad HAT en el entorno del promotor regulado.

4.2.5. Complejos remodeladores de cromatina dependientes de ATP

Los factores remodeladores de cromatina como SWI2/SNF2, ISWI/SNFL2 o WINAC, usan la energía de la hidrólisis del ATP para catalizar la movilización de nucleosomas, lo que se traduce en un cambio neto en la posición relativa del octámero de histonas frente al DNA. Se cree que este cambio facilita tanto el acceso como la función de componentes claves del aparato transcripcional. Estos complejos comprenden una subunidad con actividad ATPasa (BRG-1 o Brahma) con un motivo de unión a nucleótido muy conservado, así como otros polipéptidos como los BAFs. La presencia de la subunidad con actividad ATPasa es requerida para la activación dependiente de ligando por varios NRs, existiendo interacciones directas entre los receptores y otros componentes de estos complejos. Así, la proteína WSTF, el factor que se encuentra delecionado en el síndrome de Williams, interacciona con VDR (100) y BAF57 otro componente de estos complejos interacciona con ER en presencia de estradiol. BAF57 también interacciona con un dominio localizado en el extremo N-terminal de los coactivadores p160 (14), sirviendo por tanto de nexo entre ambos tipos de complejos. En cualquier caso, las interacciones con estas proteínas tienen como consecuencia la remodelación de los nucleosomas cercanos al sitio de unión a los NRs, necesaria para la activación transcripcional.

Los complejos remodeladores de cromatina también pueden mediar represión transcripcional por los NRs. Algunos de estos complejos, como SWI/SNF y WINAC pueden ejercer tanto funciones activadoras como represoras (64, 79, 180), otros como ISWI/SNFL parecen estar implicados solamente en activación transcripcional y por último los complejos NURD/Mi2 median represión (195).

4.2.6. El complejo TRAP/DRIP

Este complejo multiproteico denominado TRAP (proteínas asociadas al TR) (60) o DRIP (proteínas que interaccionan con el VDR) (157, 158) es análogo al complejo transcripcional de levaduras denominado "Mediator". Se trata de un gran complejo que es reclutado por diferentes clases de activadores transcripcionales como SREBP, NF-κB, VP16 o los NRs. Formado por un gran número de componentes, interacciona con distintos NRs de forma dependiente de ligando y del dominio AF-2 a través de la subunidad TRAP220/DRIP205 que contiene un dominio LxxLL idéntico al de los coactivadores p160, y atraería al receptor el resto de las subunidades (156). El complejo TRAP/DRIP es capaz de potenciar la activación transcripcional dependiente de ligando por los NRs aunque no tiene ninguna actividad enzimática

intrínseca (222). Sin embargo, algunos de sus componentes forman a su vez parte del holoenzima de la RNA-polimerasa II, con lo cual, su función podría ser la de atraer a la polimerasa al promotor diana.

No está claro si existe interacción de TRAP/DRIP con el sistema p160/CBP/PCAF. Sin embargo, se ha puesto de manifiesto que la activación transcripcional dependiente de ligando por los NRs requiere el reclutamiento tanto de los coactivadores p160 como de TRAP/DRIP (120), quedando por dilucidar si actúan independientemente o consecutivamente.

4.2.7. Otros coactivadores

Hasta la fecha se han identificado diversas proteínas con capacidad de potenciar la activación transcripcional por los NRs. Algunos de esos factores, interaccionan con los receptores de manera dependiente de ligando y requieren para ello el dominio AF-2 de los NRs. Es el caso de ARA70, específico de AR, o NCoA62. Otros coactivadores interaccionan con el dominio AF-1, como PGC-1, un coactivador que en principio se describió como específico de PPARγ y que juega un papel clave en la termogénesis adaptativa (154). Otros coactivadores como TLS, Trip-1/Sug-1 o TSC-2 pueden estar implicados en rutas de degradación, transporte nuclear o estabilidad del mensajero (63, 153). Por último, se ha identificado un coactivador de RNA para los receptores esteroideos (110), SRA, que se une exclusivamente a la región AF-1 y se detecta en grandes complejos en los que está presente SRC-1.

4.2.8. Otras modificaciones de las histonas y cofactores

Como apuntábamos anteriormente, las histonas sufren distintos tipos de modificaciones post-traduccionales que pueden determinar en gran medida el tipo de respuesta transcripcional producida en un promotor específico.

La metilación de histonas, producida por las PRMTs (proteínas arginina metiltransferasas), recientemente ha sido relacionada con la activación génica. PRMT1 y CARM1 contienen un dominio HMT (metiltransferasa de histonas) y se unen al dominio AD2 de los coactivadores p160 funcionando como coactivadores secundarios en la regulación de la expresión génica por los receptores de hormonas (35). CARM1 actúa sinérgicamente junto con CBP/p300 y los coactivadores p160 potenciando la activación transcripcional dependiente de ligando por los NRs.

Se ha sugerido la función como correguladores de varias proteínas implicadas en la proteolisis, como ubiquitina ligasas y componentes del proteasoma, ya que son reclutados *in vivo* a los promotores diana e incluso en algunos casos son requeridos para la transactivación por los NRs. A su vez, los NRs y varios tipos de correguladores son proteínas diana del proteasoma. La maquinaria de ubiquitinación y proteasoma puede ejercer un papel en el control del ensamblaje/desensamblaje de los receptores y distintos cofactores al promotor, lo cual se ha demostrado que ocurre de una manera cíclica (130, 149). La ubiquitinación de histonas puede regular a su vez la metilación, conectando la vía del proteasoma a la regulación epigenética (55).

Ubc9, el enzima conjugante del grupo SUMO, modifica no solamente a las histonas, sino a gran número de NRs y correguladores (182), lo que parece estar implicado en la estabilización de proteínas y en la localización subcelular (145).

La enzima poli (ADP-ribosa)-polimerasa 1 (PARP1) transfiere cadenas de ADP-ribosa usando como sustrato NAD^+, tanto a las histonas, como a distintos factores de transcripción, como a si misma. Se piensa que la ADP-ribosilación de histonas, podría estar implicada en la activación transcripcional por los NRs (169). PARP1 tiene efectos contrapuestos sobre la estructura de la cromatina, pudiendo tanto compactarla como descompactarla dependiendo del tipo de estímulo recibido (99). También se ha propuesto su función como corregulador actuando como plataforma de interacción con el complejo "Mediator" (146).

4.3. Represión transcripcional independiente de ligando

Además de la activación transcripcional dependiente de ligando, ciertos receptores como TR y RAR, reprimen la transcripción basal en ausencia de ligando. Se asume un modelo en el que los receptores vacíos se hallarían sobre su elemento de respuesta unidos a correpresores, que serían los responsables del estado reprimido de la transcripción. La unión del ligando revertiría el silenciamiento transcripcional y conduciría a la activación génica a través de la liberación de los correpresores y la unión de complejos coactivadores.

4.3.1. Correpresores

4.3.1.1. NCoR y SMRT

Los correpresores mejor caracterizados son las proteínas de 270 kDa que se identificaron asociadas a TR y RAR, NCoR (correpresor nuclear) (86) o RIP-13 y SMRT (mediador del silenciamiento por RAR y TR) (37) o TRAC2. Estas proteínas interaccionan con RAR y TR en ausencia de ligando, interacción que es revertida tras la unión del agonista. Tanto SMRT como NCoR ejercen su función represora a través del reclutamiento de actividades desacetilasas de histonas a los promotores regulados por los factores con los que interaccionan. Estos dos correpresores, de los que se han identificado varias isoformas (38), están relacionados tanto estructural como funcionalmente. Contienen 3 dominios represores autónomos (RDs) y un dominio de interacción con los receptores nucleares (RID) que se localiza en el extremo C-terminal de la proteína (Figura 4). El RID se compone de dos motivos (cajas CoRNR) que contienen la secuencia consenso LxxI/HIxxxI/L, la cual adopta la disposición de una hélice α anfipática (87, 137, 150). Existe una clara similitud entre este motivo y el motivo LxxLL presente en algunos coactivadores. En este sentido, el motivo CoRNR presentaría una hélice extendida en el extremo N-terminal comparado con el motivo LxxLL.

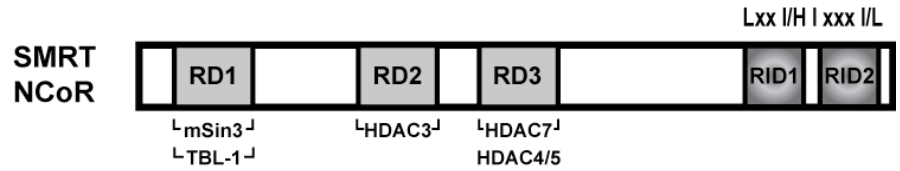

Figura 4. Estructura de los correpresores SMRT y NCoR. Se distinguen 3 dominios represores, RD1, RD2 y RD3 y los 2 dominios de interacción con los NRs (RIDs). Los RIDs contienen cada uno un motivo LxxI/HxxxI/L. El RD1 está implicado en la interacción con mSin3 a través del que se reclutan las desacetilasas de clase I. A través del mismo dominio, TBL-1 es capaz de interaccionar tanto con el correpresor como con mSin3. El RD3 es capaz de reclutar directamente las desacetilasas de clase II, HDAC4, 5 y 7. La interacción de HDAC3 se produce principalmente a través del RD2.

Se ha identificado en los receptores nucleares una caja CoR esencial para la interacción con los correpresores localizada en la H1 del LBD cercana al domino D (37, 86). Sin embargo esta caja CoR parece que no formaría parte de la superficie de interacción, sino que ayudaría al posicionamiento de otras hélices implicadas en la unión directa con los correpresores. Se ha comprobado que el motivo CoRNR de los correpresores no interacciona directamente con residuos situados en esta región, sino que se ancla al surco hidrofóbico ocupado en presencia de ligando por los motivos LxxLL del coactivador, desplazando hacia afuera a la región AF-2 debido al mayor tamaño que le confiere su extensión N-terminal (150). La unión del ligando cerraría la estructura reposicionando la H12 e impidiendo por tanto la unión del correpresor. Puesto que la superficie de interacción con correpresores solapa con la implicada en el reclutamiento de coactivadores, la unión de uno u otro tipo de correguladores al receptor es mutuamente excluyente. Recientemente se ha obtenido la estructura cristalográfica del LBD de PPARα interaccionando con un fragmento de SMRT que contiene el RID en presencia de un antagonista. En esta estructura la caja CoRNR adopta una conformación de hélice α de tres vueltas que se une a la superficie de unión de coactivadores y por tanto impide que el dominio AF-2 adopte la conformación agonista (217).

La región AF-2 desempeña un papel crucial en la disociación de correpresores por los NRs. La H12 inhibe el reclutamiento de correpresores por la mayoría de los NRs, comportándose los receptores que carecen de este dominio como represores transcripcionales constitutivos. Este es el caso de la oncoproteína v-erbA (44), versión mutante del TR y del receptor huérfano RevErb (78). La deleción de la H12 permite a RXR, incapaz de unir correpresores y transrreprimir, la interacción con éstos y la represión génica (226). En el caso de TR y RAR, la mutación o la deleción del dominio AF-2 potencia la interacción con correpresores y dificulta su liberación tras unirse el ligando (118). Por tanto, la unión del ligando por si misma parece no ser suficiente para la disociación de los correpresores, siendo la H12 determinante en esta liberación.

A pesar de que los receptores esteroideos no parecen reprimir la transcripción en ausencia de ligando y de que tampoco se ha detectado interacción con correpresores en presencia de un ligando agonista, se ha observado un claro reclutamiento de correpresores cuando los receptores están ocupados por antagonistas (181, 228). Dicho reclutamiento junto con la competencia con los agonistas por la unión al receptor, sería el responsable de la disminución de la respuesta transcripcional causada por los

antagonistas. La unión del antagonista al receptor impide al dominio AF-2 adoptar la conformación agonista normal. Se induciría un posicionamiento diferente de la H12 ocupando ésta el sitio de unión de los coactivadores y formándose a su vez una superficie adecuada de unión de correpresores (24, 178).

La estequiometría parece ser también determinante en la unión de los correpresores a los NRs. Por ejemplo, los correpresores son capaces de unirse a homo o heterodímeros pero no a monómeros de TR sobre el DNA. Por otro lado, la unión de correpresores está claramente controlada por efectos estéricos derivados de la unión de los NRs al DNA. Es por ello que pese a que la mayoría de receptores son capaces de unir correpresores en solución, el reclutamiento se hace mucho más restrictivo cuando están unidos sobre el DNA. Esto explica porqué receptores como PPAR no se comportan como silenciadores transcripcionales cuando se encuentran unidos a su elemento de respuesta a pesar de que son capaces de interaccionar tanto con SMRT como con NCoR en solución (224).

4.3.1.2. Otros correpresores

Además de SMRT y NCoR, existen otra serie de proteínas que actúan como correpresores de los NRs. SUN-CoR pese a no presentar homología con NCoR o SMRT, potencia la represión transcripcional mediada por TR y RevErb. También es capaz de interaccionar con SMRT y NCoR, por lo que se ha propuesto que podría actuar como un componente adicional del complejo implicado en la represión transcripcional por los receptores huérfanos o en ausencia de ligando (223). Otro correpresor que carece de homología significativa con SMRT y NCoR es Alien. Se ha descrito su interacción con receptores como TR y DAX-1 pero no con RAR o RXR. Todo esto lleva a considerarlo como perteneciente a una clase distinta de correpresores que interaccionarían de una manera sensible al tipo de hormona implicada (50).

Se han descrito varios casos de correpresores que son reclutados por los NRs en presencia de ligando. RIP140 se identificó inicialmente como un coactivador (31), que interaccionaba de manera dependiente de agonista con los NRs a través de motivos LxxLL (81). Sin embargo, más adelante se observó que actuaba como un correpresor que competía con los coactivadores p160 por la unión a los NRs, bloqueando el efecto de los coactivadores *in vivo*. Actualmente se cree que actuaría como un factor regulador influenciando la unión de coactivadores probablemente por un mecanismo de competición por los NRs (197). A pesar de tener escasa homología, se ha descrito un correpresor que presenta gran paralelismo funcional con RIP140. LCoR interacciona en presencia de hormona con ER y estimula la represión de la transcripción mediada por varios NRs a través de un único motivo LxxLL indispensable para la interacción con éstos (59). Otros casos de correpresores que ejercen sus funciones de manera dependiente de agonista son los factores REA que se asocia a ER (47) y el recientemente identificado PRAME, que media represión por RAR (53).

4.3.2. Desacetilasas de histonas

La represión transcripcional producida por la unión de correpresores a los NRs parece estar mediada por el reclutamiento de desacetilasas de histonas o HDACs al promotor diana. HDAC1 y HDAC2 se encuentran formando grandes complejos multiproteicos asociadas a los factores Sin3. mSin3 (forma presente en mamíferos) es una proteína adaptadora de gran tamaño y con múltiples dominios que forman la estructura básica sobre la que se ensamblan el resto de componentes del complejo (7). Los complejos mSin3 – HDAC son estables y abundantes en la célula, no suponiendo ningún factor limitante para su reclutamiento por los correpresores. Las proteínas Sin3 contienen 4 repeticiones imperfectas de un motivo PAH que les permite interaccionar con los correpresores. SMRT y NCoR interaccionan con mSin3 a través del primer dominio represor (RD1) localizado en el extremo N-terminal de estos correpresores (82, 136). Ya que la interacción entre mSin3 y los NRs no es directa, la función de correpresores como SMRT y NCoR podría ser la de conectar los receptores con los complejos HDAC.

Se pensaba que los correpresores actuaban exclusivamente a través del reclutamiento indirecto de HDAC1 y HDAC2 (desacetilasas de clase I) a través de mSin3. Sin embargo se ha demostrado que los correpresores son capaces de interaccionar a través del dominio represor RD3 con las llamadas desacetilasas de clase II. Estas desacetilasas (HDAC4, 5 y 7) se encuentran asociadas a los correpresores en complejos que no contienen mSin3 ni HDAC1 (88). Por tanto, un único correpresor podría usar distintos dominios represores para reclutar desacetilasas de clase I a través de mSin3 o bien desacetilasas de clase II de manera independiente de mSin3 (96). Se ha identificado un último complejo con actividad desacetilasa de histonas. Contiene la desacetilasa HDAC3 y la proteína TBL1, que interacciona con la histona H3. TBL1 se ancla a HDAC3 a través del correpresor potenciando la represión por los NRs (74, 114, 207, 220). SMRT y NCoR funcionan además como cofactores activadores de HDAC3 a través un dominio activador de desacetilasas (DAD) situado en el extremo N-terminal de estos correpresores. Así SMRT y NCoR formarían parte integrante del enzima activo HDAC3 (73).

Todas estas observaciones sugieren que la compactación de la cromatina debida a la desacetilación de las histonas a través de la acción de los complejos reclutados a los NRs por los correpresores estaría implicada en la represión transcripcional por los receptores no esteroideos en ausencia de ligando o por los esteroideos en presencia de antagonistas. La unión del agonista permitiría la liberación de los correpresores y el reclutamiento de coactivadores que conduciría a la activación de la transcripción.

4.4. Intercambio cíclico de factores activadores y represores en la regulación transcripcional por los receptores nucleares

La unión del ligando es el evento crucial que dispara el cambio desde la represión a la activación transcripcional por los NRs. Numerosos estudios han tratado de elucidar el orden de reclutamiento de los distintos cofactores que median tanto la activación como la represión génica. Se trata de un reclutamiento dinámico de cofactores asociado a la unión de los NRs al DNA, caracterizada por ciclos

de reclutamiento y liberación (159). Se postula que la llegada del ligando desencadenaría la unión de distintas clases de coactivadores que actuarían ordenadamente. La unión de un tipo de estos complejos facilitaría el reclutamiento del siguiente tipo de coactivador requerido (Figura 5). Primeramente se reclutarían complejos con actividad HAT y HMT que provocarían la acetilación y metilación de la cromatina y por tanto su descompactación. Los complejos remodeladores de cromatina podrían ser reclutados seguidamente o incluso en los estadios más iniciales en un contexto de cromatina altamente condensada (149). Ya que distintos coactivadores se unen a las mismas regiones de los NRs, se produciría una competencia por la unión a los receptores. Así los p160 se disociarían sufriendo acetilación por factores como CBP/p300 o siendo directamente dirigidos a degradación por el proteasoma lo que permitiría la unión de otros coactivadores como el complejo TRAP/DRIP. Esto reclutaría la polimerasa y la maquinaria de transcripción basal (1). Tras el primer ciclo de activación génica, los complejos remodeladores de cromatina mediarían el cambio hacia la represión asociándose con desacetilasas de histonas. A su vez los correpresores basales como SMRT y NCoR reclutarían también este tipo de actividades que desembocaría en un estado de compactación de la cromatina y represión transcripcional. Por último, la maquinaria de ubiquitinación promovería la degradación por el proteasoma de los complejos correpresores, necesaria para la producción de un nuevo ciclo de activación transcripcional (130, 148).

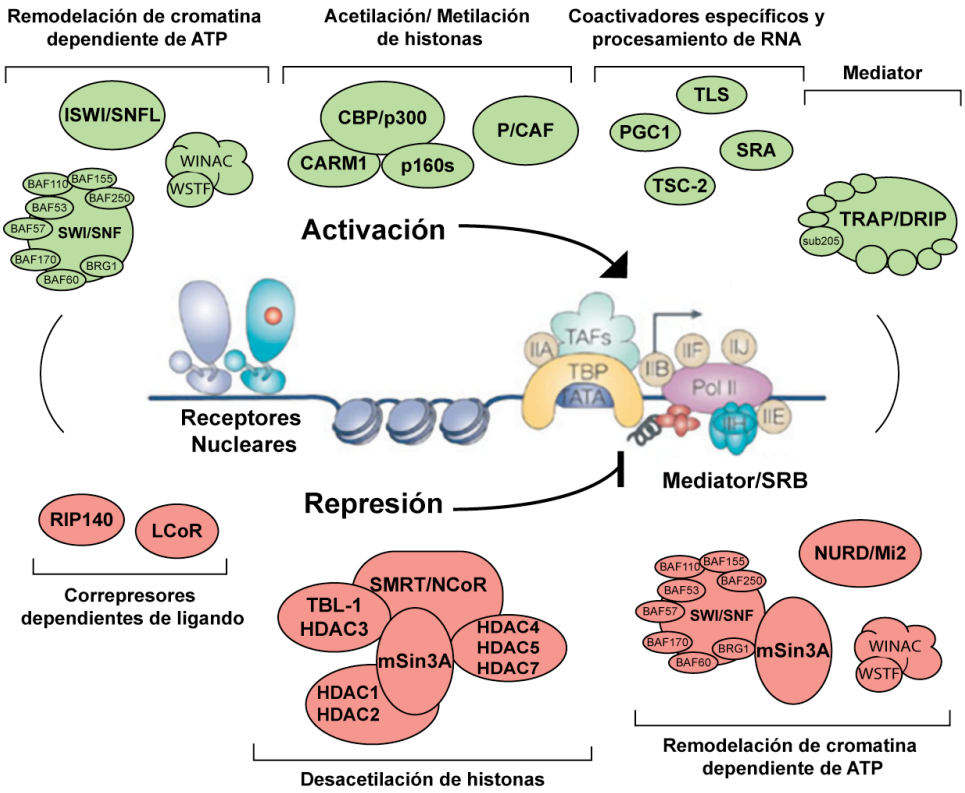

Figura 5. Transición de la represión a la activación transcripcional mediada por los receptores nucleares. Los complejos coactivadores (color verde) que median la activación transcripcional comprenden factores remodeladores de cromatina dependientes de ATP, acetiltransferasas de histonas, arginina-metiltransferasas como CARM, así como el complejo "Mediator" que sirve de nexo con la RNA-polimerasa y la maquinaria de transcripción basal. Los complejos correpresores (color rojo) a su vez incluyen correpresores basales como SMRT y NCoR que funcionan como plataformas donde se anclan distintos complejos con actividad desacetilasa de histonas y correpresores específicos como RIP140 y LCoR capaces de producir represión en presencia de ligando. También se incluyen entre los factores implicados en la represión transcripcional complejos remodeladores de cromatina dependientes de ATP similares a los implicados en activación, o específicos de funciones represoras.

4.5. Represión transcripcional dependiente de ligando

4.5.1. Mecanismos pasivos

Los NRs también pueden reprimir la expresión génica de manera dependiente de ligando. En ciertos casos la represión puede ser debida a una inhibición pasiva causada por la formación de dímeros transcripcionalmente inactivos. Así, la dimerización de un receptor funcional con receptores mutados o truncados puede dar origen a la formación de dímeros inactivos que bien no se unen a DNA, o que aunque se unan sean transcripcionalmente inactivos. En el caso de los receptores que forman heterodímeros, un receptor inactivo puede inhibir la acción no solamente de su receptor nativo sino también de otros receptores que comparten la misma pareja heterodimérica (11).

La inhibición transcripcional por los NRs puede también producirse por interferencia debida a la superposición del HRE con otros elementos del DNA que unen otros activadores transcripcionales. En este caso la unión del complejo hormona-receptor al DNA desplazaría a otros factores de transcripción de sus sitios de unión (106, 125).

Por último, los NRs pueden inhibir respuestas transcripcionales de genes que no contienen un HRE a través de la regulación de la actividad de los factores de transcripción que se unen a otros elementos del promotor regulado, un mecanismo al que se denomina "**transrrepresión**". En este caso la inhibición se produciría por interacciones directas proteína-proteína con otros factores de transcripción (132, 230) y/o por competición por coactivadores comunes que se requieren para la activación de la transcripción por ambos, como el CBP/p300 (94, 103, 116, 174), aunque actualmente se postulan distintos mecanismos para este tipo de fenómenos (145).

4.5.2. Mecanismos activos. Elementos de respuesta negativos

Existen HREs negativos que median una represión activa en presencia de ligando. Son particularmente importantes en los mecanismos de autoinhibición implicados en la síntesis hormonal. Así, los glucocorticoides regulan negativamente la expresión del gen de la pro-opiomelanocortina (POMC) y las hormonas tiroideas inhiben la transcripción del gen de la hormona tirotropa (TSH) (22, 51). En los promotores de estos genes se encuentran estos elementos negativos en los que generalmente el receptor vacío activa la transcripción y la unión del ligando revierte dicha estimulación. Normalmente los elementos negativos se localizan muy próximos al sitio de inicio de la transcripción, algunos por debajo de la caja TATA e incluso en la región 3' no traducida del gen (13, 18, 147, 167). Paradójicamente se ha descrito la implicación de correpresores en la activación basal mediada por TR en ausencia de ligando, resultando la sobreexpresión de SMRT o de NCoR en un incremento de la actividad basal del promotor de TSH (189).

Objetivos

Objetivos

Los objetivos fundamentales para la realización de esta tesis han sido:

1. Estudio de función del RXR y su ligando en la regulación de la transcripción mediada por los heterodímeros TR/RXR y VDR/RXR.

A) Estudio de la regulación del gen de la prolactina de rata por los rexinoides y las hormonas tiroideas.

- Análisis del reclutamiento de coactivadores y correpresores en respuesta a los ligandos del heterodímero TR/RXR.
- Identificación de los dominios funcionales de los receptores implicados.
- Consecuencias de cambios en los niveles de coactivadores y correpresores sobre la transactivación por triyodotironina (T3) y ácido 9-*cis*-retinoico (9-*cis*RA).

B) Papel del RXR y sus ligandos en la unión de coactivadores y la transactivación por el heterodímero VDR/RXR.

- Contribución de los dominios funcionales de los receptores implicados.
- Papel del RXR y su ligando en la activación transcripcional del gen *cyp*24 por la vitamina D.
- Cooperación de los ligandos de VDR y RXR en la diferenciación de células de cáncer de colon.

C) Estudio del reclutamiento de los correpresores SMRT y NCoR por el heterodímero VDR/RXR.

- Caracterización del reclutamiento de correpresores por el heterodímero tanto *in vitro* como sobre el promotor del gen *cyp*24.
- Papel de los ligandos de ambos miembros del heterodímero en el reclutamiento y la liberación de los correpresores.
- Identificación de los dominios de VDR y RXR implicados en el reclutamiento de correpresores.
- Función de los correpresores endógenos en la activación transcripcional mediada por vitamina D.

2. Caracterización *in vitro* e *in vivo* de diversos análogos sintéticos de la vitamina D y sus efectos sobre la transactivación y transrrepresión en distintos tipos celulares.

Resultados

A PERMISSIVE RETINOID X RECEPTOR/THYROID HORMONE RECEPTOR HETERODIMER ALLOWS STIMULATION OF PROLACTIN GENE TRANSCRIPTION BY THYROID HORMONE AND 9-*cis*-RETINOIC ACID

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Introducción al Capítulo 1

Las hormonas tiroideas desempeñan un papel fundamental en la regulación del desarrollo, crecimiento y metabolismo de los organismos superiores. La mayoría de las acciones celulares de estos compuestos están mediadas a través de la unión a los receptores nucleares de hormonas tiroideas (TRs), factores de transcripción dependientes de ligando que se unen al DNA en forma de heterodímeros con el RXR. En este trabajo describimos por primera vez como un gen natural, el de la prolactina de rata, puede estar regulado a través del mismo elemento de respuesta a hormonas tiroideas (TRE), tanto por las mismas, como por el ligando natural del RXR, el 9-*cis*RA. Hemos observado que no sólo la T3 sino también el 9-*cis*RA aumentan la expresión del RNA mensajero de prolactina en células somato-lactotrofas GH4C1 a través de la unión de heterodímeros TR/RXR a un TRE positivo localizado en el "enhancer" distal del gen. Dicho elemento se configura como DR4 y está localizado entre los nucleótidos -1551 y -1566 del promotor. La unión de heterodímeros TR/RXR al TRE de la prolactina media la estimulación del gen por ligandos de los dos miembros de la pareja heterodimérica ya que la expresión de TR en células hipofisarias 235-1, carentes de este receptor, confiere capacidad de respuesta no solamente a T3, sino también a 9-*cis*RA. Estos resultados sugieren que el RXR no actuaría como un compañero silencioso de TR en la estimulación del gen de la prolactina de rata. Prueba de ello es que el heterodímero unido al TRE es capaz de reclutar coactivadores en respuesta a los dos ligandos de la pareja. Además, la combinación de ligandos produce un efecto cooperativo en el reclutamiento de coactivadores que se corresponde con el observado en la activación transcripcional del gen de prolactina. Existe una íntima comunicación entre los dos receptores que conforman el heterodímero ya que la deleción del dominio AF2 del TR inhibe tanto el reclutamiento de coactivadores como la activación transcripcional en respuesta al ligando del RXR. Esta comunicación hace sin embargo que el tratamiento con 9-*cis*RA sea capaz de revertir el efecto deletéreo de una mutación puntual (E401Q) en dicha región del TR que causa pérdida de reclutamiento de coactivadores y por tanto de respuesta transcripcional a T3. Es particularmente interesante este hecho ya que esta mutación está presente en algunos pacientes con síndrome de resistencia a hormonas tiroideas. Así este tipo de receptores defectuosos podrían recuperar su capacidad de respuesta a la hormona cuando ésta se administra combinada con rexinoides. Finalmente, analizamos el papel que los distintos correguladores pueden ejercer sobre la respuesta transcripcional al ligando del RXR. Según el tipo celular se obtenían distintas respuestas al 9-*cis*RA, desde similares a las producidas por la T3, como ocurre en células Hela o GH4C1, hasta ausencia de respuesta como ocurre en células CV-1. La sobre-expresión de coactivadores en estas últimas es capaz de permitir la respuesta a 9-*cis*RA, lo que indicaría que se puede obtener transcripción en respuesta a agonistas del RXR en células que expresen altos niveles de coactivadores. Por su parte, la sobre-expresión de correpresores en células hipofisarias disminuye la respuesta a ambos ligandos, pero aún se observa un sinergismo de acción de los mismos cuando son combinados. Estas observaciones ponen de manifiesto que la permisividad de este tipo de heterodímeros podría lograrse en ambientes celulares con una proporción de coactivadores y correpresores adecuada. En resumen, nuestros resultados demuestran por

primera vez que los heterodímeros TR/RXR, tradicionalmente considerados como no permisivos, pueden mediar la estimulación de la transcripción de un gen natural por agonistas del RXR.

La contribución técnica de la alumna a este trabajo fundamentalmente radica en la realización de ensayos EMSA con distintos receptores, coactivadores y correpresores, bien salvajes o mutantes, y su caracterización funcional en ensayos de transfección transitoria en diversas líneas celulares. Por su parte la alumna ha contribuido al diseño experimental y posterior discusión de los resultados mostrados en este artículo.

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A Permissive Retinoid X Receptor/Thyroid Hormone Receptor Heterodimer Allows Stimulation of Prolactin Gene Transcription by Thyroid Hormone and 9-*cis*-Retinoic Acid

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Heterodimers of the retinoid X receptor (RXR) with the thyroid hormone receptor (TR) are considered to be nonpermissive. It is believed that within these complexes RXR acts as a "silent partner." We demonstrate here that a permissive heterodimer mediates stimulation of prolactin expression by the thyroid hormone T3 and by 9-*cis* retinoic acid (9-*cis*-RA). A response element located in the prolactin distal enhancer mediates transactivation by both ligands in pituitary cells, and RXR recruits coactivators when bound to this element as a heterodimer with TR. Furthermore, transcription by the RXR agonist can be obtained in CV-1 cells only after overexpression of coactivators, and overexpression of corepressors inhibits the response in pituitary cells. Thus, cell type-specific differences in coregulator recruitment can determine the cellular response to both ligands. Coactivator recruitment by 9-*cis*-RA requires the ligand-dependent transactivation domains (AF-2) of both heterodimeric partners. Interestingly, the presence of the RXR ligand can overcome the deleterious effect of the AF-2 mutation E401Q on association with coactivators and transactivation. These results demonstrate an unexpected role for RXR in TR signaling and show that in particular cellular environments this receptor can act as a "nonsilent" partner of TR, allowing stimulation by RXR agonists.

The actions of the thyroid hormone triiodothyronine (T3) are mediated by binding to nuclear thyroid hormone receptors (TRs). TRs are ligand-dependent transcription factors which regulate transcription by binding to T3 response elements (TREs) in target genes (53). TREs are composed of at least two copies of the consensus motif PuG/TGTCA, configured as a palindrome, an inverted palindrome, or a direct repeat normally separated by four intervening nucleotides (DR4). TRs, as well as many other nuclear receptors, bind DNA preferentially as heterodimers with retinoid X receptors (RXRs). Heterodimerization strongly increases binding to the TRE and transcriptional activity. Therefore, RXR plays a dual role in nuclear receptor signaling. On one hand, it can bind to its own response element, a DR1, as a homodimer and activate transcription in response to its ligand 9-*cis*-retinoic acid (9-*cis*-RA) (21, 27), and on the other hand it serves as a partner for other nuclear receptors (31).

The existence of two types of nuclear receptor heterodimers, nonpermissive and permissive, has been described. Permissive heterodimers can be indistinctly activated by ligands of either RXR or its partner receptor and are synergistically activated in the presence of both ligands (1). However, in nonpermissive heterodimers the ligand-induced transcriptional activities of RXR are suppressed, and it is believed that formation of the heterodimer actually precludes the binding of ligand to RXR (15). Thus, in these complexes, RXR is said to be a "silent partner." TRs as well as the receptors for vitamin D or for retinoic acid (RARs) were thought to be nonpermissive.

The effects of TRs, as well as other nuclear receptors, on transcription are mediated through recruitment of coregulators. TRs bind corepressor factors and actively repress target gene expression in the absence of ligand. Corepressors are found within multicomponent complexes, which contain histone deacetylase activity (22). Upon ligand binding the receptors undergo a conformational change, which allows the recruitment of multiple coactivator complexes through a ligand-dependent transcriptional activation function (AF-2) located in helix 12 at the C terminus in the ligand binding domain (2). Some of these proteins are chromatin-remodeling factors, others (such as CBP/p300 and the p160 coactivators) possess histone acetylase activity, and others (such as the TRAP/DRIP complex) may interact directly with the basic transcriptional machinery. These coactivators cause chromatin decompaction, RNA polymerase II recruitment, and transcriptional activation (16, 24, 32, 39, 40).

Results arguing against the current silent-partner model for RXR in the RXR/TR heterodimer have been recently obtained with a derepression assay system (28). According to this model RXR would bind ligand and this binding would lead to dissociation of corepressors from TR, thus modulating heterodimer activity. Since RXR was believed not to bind ligand, it was assumed that coactivators could not be recruited to a nonpermissive heterodimer in response to 9-*cis*-RA. However, recent data indicate that RXR can recruit coactivators as a heterodimer with RAR. Lack of autonomous transcription on binding of the RXR agonist would be due to the fact that in the usual cellular environment corepressors do not dissociate from RAR and they prohibit coactivator access because corepressor

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binding and coactivator binding are mutually exclusive (18). This model predicts that transcription by RXR agonists (rexinoids) could be obtained under some conditions, for instance, in cells expressing high coactivator levels, although evidence for natural genes regulated in this manner had not been obtained.

The rat prolactin (PRL) gene provides an excellent model for the study of cell-specific and multihormonal regulation. Multiple hormones, growth factors, and oncogenes act in conjunction with the pituitary-specific transcription factor GHF-1/Pit-1 to regulate PRL gene expression in the lactotroph cells of the anterior pituitary. Transcription of the PRL gene is governed by two domains, a proximal promoter and a distal enhancer (located between bp −1500 and −1800), both containing binding sites for GHF-1/Pit-1. PRL-producing rat pituitary cell lines expressing high levels of different members of the nuclear receptor superfamily are available. Several nuclear receptors, among them estrogen receptors (ERs) (12), peroxisome proliferator-activated receptors (46), vitamin D receptors (5), and glucocorticoid receptors (41), are known to play an important role in PRL gene transcription in these cells. In addition, it has been reported that the thyroid hormone regulates both positively and negatively PRL gene transcription in rat pituitary cell lines. Thus, it was reported that T3 inhibits and stimulates PRL transcription in GH1 cells and GH4C1 cells, respectively, through sequences located in the proximal promoter (44). In contrast, it has been found that in GH3 cells a region close to the ER response element (ERE) in the distal enhancer mediates stimulation, whereas sequences contained in the proximal promoter mediate inhibition by T3 (11). However, a detailed analysis of these sequences has not been performed, and the role of RXR in this regulation has not been analyzed.

In this work we have examined the role of RXR/TR heterodimers in PRL gene expression. We find that not only T3 but also 9-*cis*-RA increase PRL transcripts in GH4C1 cells. We demonstrate binding of RXR/TR heterodimers to a positive TRE, configured as a DR4, in the distal enhancer between nucleotides −1551 and −1566. This TRE mediates stimulation of the PRL gene by ligands of both heterodimeric partners in transient-transfection assays. Furthermore, expression of TR in pituitary 235-1 cells lacking this receptor confers responsiveness not only to T3 but also to 9-*cis*-RA. The TRE does not mediate regulation by 9-*cis*-RA in CV-1 cells, but overexpression of coactivators allows stimulation by this ligand, fulfilling the prediction that transcription by RXR agonists can be obtained in cells expressing high coactivator levels. These results strongly suggest that RXR does not act as a silent partner in the RXR/TR heterodimer to stimulate PRL gene transcription. This hypothesis is further proved by the finding that the TRE-bound heterodimer can recruit p160 coactivators or the TRAP205 subunit of the DRIP/TRAP complex in response to either agonist. Interestingly, deletion of the TR AF-2 domain inhibits coactivator recruitment, as well as activation of the TRE-containing construct, by the RXR ligand. Furthermore, the presence of the RXR ligand overcomes the deleterious effect of a point mutation (E401Q) in the TR AF-2 domain on association with coactivators and transcriptional stimulation. These results show that different conformations of the heterodimer can be induced by both agonists and that binding to either agonist results in a linked conformational change in the other receptor subunit. Therefore, our results prove for the first time that RXR/TR heterodimers, previously regarded as nonpermissive, can mediate stimulation of transcription of a natural gene by the RXR agonist 9-*cis*-RA.

MATERIALS AND METHODS

RNA extraction and hybridization. For the experiments the cells were incubated for 24 h in a medium containing a hormone-stripped serum and treated for 48 h with different ligands. Total RNA was used for Northern blot analysis with a cDNA probe for rat PRL as described previously (5). The RNA was stained with 0.02% methylene blue to detect rRNA as a control for loading.

Plasmids. Reporter plasmids containing different fragments of the rat PRL 5′-flanking region have been previously described (5, 29). The mutated constructs were obtained with the *Pfu* Turbo DNA polymerase (Stratagene), by using the oligonucleotide 5′-TGCTTTGG**TC**TCAGAAGA**TT**CAG-3′ (boldface indicates mutated bases). The mutations were confirmed by sequencing. Oligonucleotides containing sequences −1551 to −1573 and −1551 to −1593 of the rat PRL distal enhancer were cloned upstream of the thymidine kinase promoter into pBL-CAT2, from which an AP-1-like sequence, which could mask some promoter responses, had been deleted by digestion with *Aat*II and *Nar*I. Expression vectors for wild-type and mutant RXRα and chick TRα have been previously described and were cloned in pSG5 (2, 3). Expression vectors for TIF-2, SRC-1, ACTR, DRIP205, SMRT, and NcoR (6, 7, 20, 39, 47) were cloned in the same vector. The glutathione *S*-transferase (GST)–ACTR, GST–TIF-2, GST–SRC-1, GST-DRIP205, and GST-SMRT vector constructs code for protein fragments containing the nuclear receptor-interacting domains of these proteins. The His-tagged nuclear receptor-interacting domain of TIF-2, as well as this domain with mutations in box II (M2) and box III (M3) have also been described (18).

Transfections. HeLa and CV-1 cells were transfected by calcium phosphate coprecipitation as described previously (46), typically with 5 μg of reporter. GH4C1 and 235-1 cells were transfected by electroporation with 15 μg of reporter plasmids as previously described (17, 42). After transfection cells were plated in medium containing hormone-stripped serum; after an overnight incubation, cells were shifted to serum-free medium and treatments were started. When appropriate, the reporter plasmid was cotransfected with the amounts of expression vectors for the receptors or coregulators indicated in the figure legends, and in this case equivalent amounts of empty vectors were used. Experiments were performed with triplicate cultures, and each experiment was repeated at least three times. Data are represented as means ± standard deviations.

Gel retardation assays. Oligonucleotides corresponding to the PRL TRE (5′-TGCTTTGGGGTCAGAAGAGGCAG-3′) and to a consensus DR4 element (5′-AGCTCAGGTCACAGGAGGTCAG-3′) were used in the assays. Wild-type and mutant TR and RXR coding sequences cloned in pSG5 were used for in vitro transcription and translation with TNT Quick (Promega). Assays were performed as previously described (5, 35, 46), with 1 μl of each receptor subunit in the presence and absence of 400 to 600 ng of the GST-fused coactivators, 450 ng of His-tagged TIF-2, or 1.5 μg of GST-SMRT.

RESULTS

T3 and 9-*cis*-RA stimulate PRL gene transcription. Figure 1A shows that 9-*cis*-RA increases PRL mRNA levels in pituitary GH4C1 cells and strongly potentiates the stimulatory effect of T3. In addition, 9-*cis*-RA and estrogen were similarly potent in inducing the levels of PRL transcripts in these cells. The combined effects of T3 and 9-*cis*-RA in transient-transfection assays with a reporter plasmid containing 5′-flanking sequences of the rat PRL gene are shown in Fig. 1B. Physiological concentrations of T3 caused a dose-dependent stimulation of the activity of this construct, and, in parallel with the cooperation shown in Fig. 1A, this response was further increased in the presence of 9-*cis*-RA, which by itself also increased reporter activity.

PRL sequences involved in regulation by T3 and 9-*cis*-RA. The influence of T3 and 9-*cis*-RA on PRL reporter plasmids

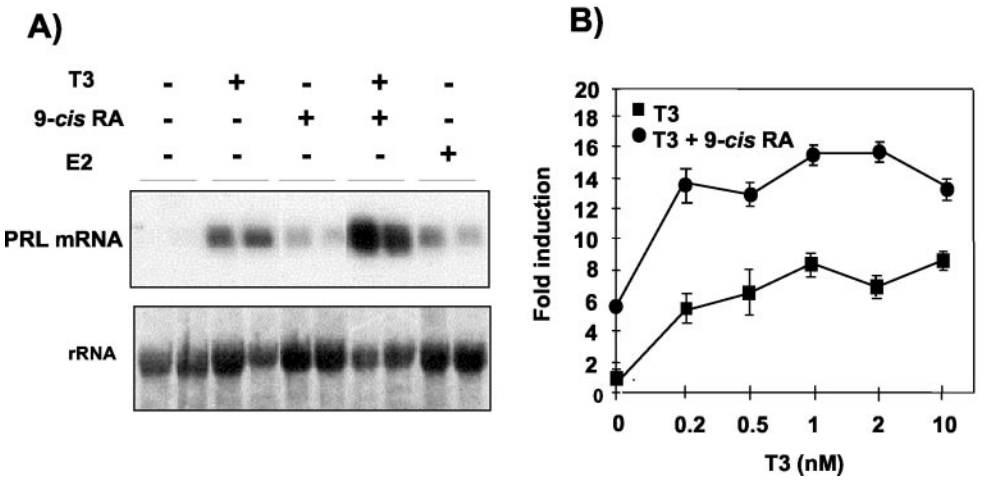

FIG. 1. T3 and 9-*cis*-RA stimulate PRL gene expression in rat pituitary GH4C1 cells. (A) Northern blot analyses were carried out with RNA from control cells and cells incubated for 48 h with 5 nM T3, 1 μM 9-*cis*-RA, or 100 nM estradiol (E2), as indicated. The blot was hybridized with a labeled cDNA probe for rat PRL. (Bottom) 18S rRNA. (B) GH4C1 cells were transfected with a PRL promoter construct containing the distal enhancer fused to the sequence from −422 to +34, and luciferase activity was determined after 48 h in untreated cells and in cells treated with increasing concentrations of T3 in the presence and absence of 1 μM 9-*cis*-RA. The data are expressed relative to those found in the untreated cells.

containing only the proximal promoter, only the distal enhancer, or both was also analyzed. As shown in Fig. 2A, T3 caused a strong induction of the activity of a plasmid containing the distal enhancer (−1831 to −1530) fused to sequence −422 to +34. This construct was stimulated by 9-*cis*-RA to a similar extent. Both ligands also increased the activity of a plasmid in which the region between nucleotides −422 and −78, which contains the proximal GHF-1/Pit-1 binding sites, has been deleted. However, neither T3 nor 9-*cis*-RA stimulated the activity of plasmids lacking the distal enhancer, showing that this region contains the sequences responsible for induction by both ligands. Inspection of the PRL distal enhancer suggested the existence of a putative TRE next to the ERE, with the configuration of a direct repeat separated by 4 nucleotides (DR4). Figure 3B shows that an oligonucleotide containing sequences between −1573 and −1551 does not bind the α isoform of TR, RAR, or RXR alone but binds strongly RXR/TR heterodimers. In contrast, with RXR/TR heterodimers, neither RXR homodimers nor RXR/RAR heterodimers bind this DNA motif with high affinity.

A common hormone response element mediates regulation of PRL gene transcription by T3 and 9-*cis*-RA. To test the

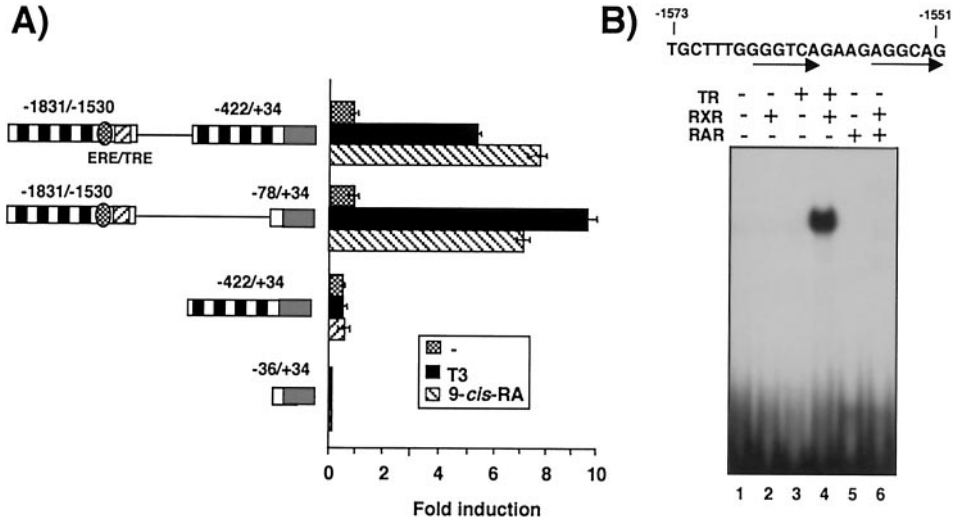

FIG. 2. The PRL distal enhancer contains an RXR/TR binding site. (A) PRL luciferase reporter constructs containing the indicated combinations of the proximal promoter (−422/+34), the distal enhancer (−1530/−1831), or basal sequences (−36/+34) were transfected into GH4C1 cells, and reporter activity was determined after 48 h in control cells and in cells treated with 5 nM T3 or 1 μM 9-*cis*-RA. Results are expressed relative to the values obtained in the untreated cells transfected with the longest construct. The TRE is depicted as a shaded rectangle adjacent to the ERE. Black boxes, binding sites for GHF-1/Pit-1. (B) Mobility shift assays with in vitro-translated TRα, RXR, and RAR (1 μl) and an oligonucleotide encompassing the sequence from −1551 to −1573 of the PRL distal enhancer.

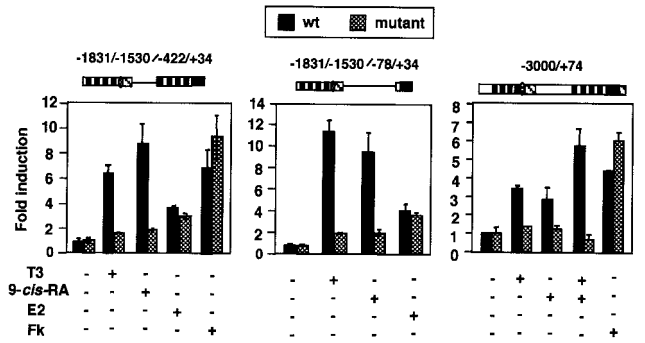

FIG. 3. The PRL TRE mediates regulation by T3 and 9-*cis*-RA. The TRE was mutated in the PRL constructs indicated at the top. The wild-type (wt) and mutant plasmids were transfected into GH4C1 cells, and reporter activity was determined after 48 h of incubation with 5 nM T3, 1 μM 9-*cis*-RA, 10 μM forskolin (Fk), or 100 nM estradiol (E2). Results are expressed as factors by which induction exceeded that obtained in the corresponding control untreated cells.

functionality of the RXR/TR binding site in the distal enhancer of the PRL gene, this element was mutated in the context of the reporter constructs shown in Fig. 3. The inserted mutation affected both hemisites of the response element and abolished binding of RXR/TR in gel retardation assays (data not shown). In all cases, mutation of this motif abolished the response to T3 and, interestingly, also blocked stimulation by 9-*cis*-RA. The effect of this mutation was specific for these ligands, since the response to estradiol was not affected. Stimulation by forskolin, which is mediated by proximal promoter sequences, was not affected either.

The finding that the same element mediates regulation by T3 and 9-*cis*-RA could be explained if RXR could act as a nonsilent partner of TR to mediate PRL gene transcription in pituitary cells. This is suggested by the finding that the TRE binds neither RXR homodimers nor RXR/RAR heterodimers. To further dismiss participation of these receptor complexes in PRL stimulation by 9-*cis*-RA, the influence of different retinoids on transient-transfection studies with the PRL promoter was determined. For this purpose, besides 9-*cis*-RA, a natural ligand that binds with similarly high affinity to both RXR and RAR, we used all-*trans*-RA, which shows a higher affinity for RAR; the RAR-selective agonist TTNPB; and the rexinoid LG100268, a specific agonist for RXR homodimers. Figure 4 shows that, compared with 9-*cis*-RA, all-*trans*-RA displays a markedly reduced ability to stimulate the PRL construct. In addition, TTNPB was unable to induce a response by itself or to cooperate with T3, demonstrating that RAR is not involved in regulation of the PRL gene by 9-*cis*-RA. TTNPB is active in pituitary cells, since this RAR-selective retinoid stimulated the activity of the RARβ2 promoter, which is mediated by RXR/RAR heterodimers (25), with the same potency as all-*trans*-RA and 9-*cis*-RA (not illustrated). Furthermore, the RXR-selective ligand increased reporter activity, although it was less effective than 9-*cis*-RA Taken together these results strongly suggest that a permissive RXR/TR heterodimer could mediate stimulation of the PRL gene by T3, 9-*cis*-RA, or both. However, our results are also compatible with the existence of a different permissive heterodimer of RXR with another still-unidentified receptor.

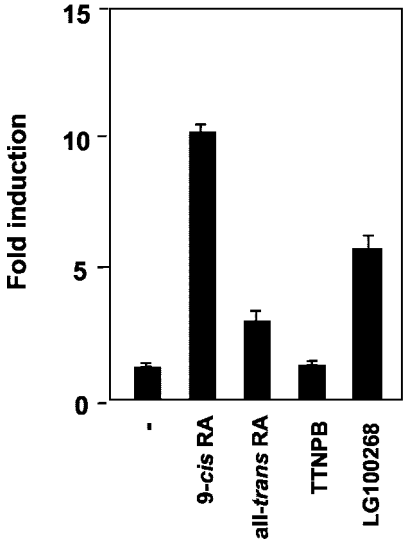

FIG. 4. The effect of 9-*cis*-RA is mediated by RXR. The PRL construct containing the distal enhancer was transfected into GH4C1 cells, and luciferase activity was measured after 48 h of incubation in the presence of 1 μM concentrations of the compounds indicated.

To directly test whether TR is required for the response to 9-*cis*-RA, we used the lactotroph 235-1 cell line, which expresses very low TR levels but which shows retinoid responses (14, 42). As shown in Fig. 5A, incubation with T3, 9-*cis*-RA, or the combination of both did not increase PRL transcription in 235-1 cells. In contrast, when the PRL construct was cotransfected with an expression vector for TR, a response to T3, although weaker than that observed in GH4C1 cells, was observed. Strikingly, 9-*cis*-RA caused a similar stimulation, and the two ligands were able to cooperate to stimulate reporter

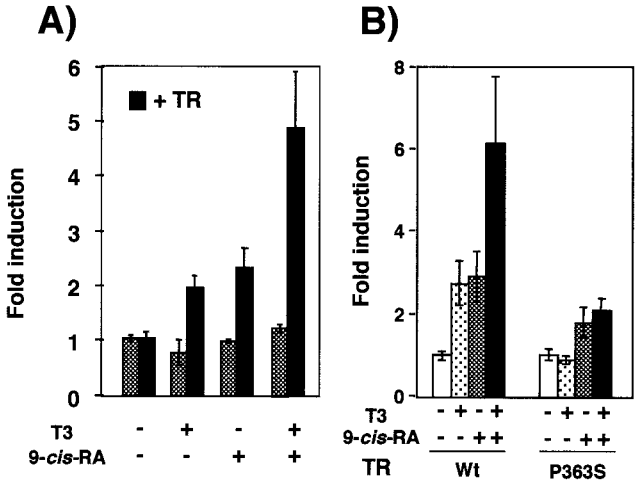

FIG. 5. Expression of TR confers responsiveness to both T3 and 9-*cis*-RA in 235-1 cells. (A) The PRL construct was transfected into pituitary 235-1 cells together with 35 μg of an expression vector for TR or with the same amount of a noncoding vector. (B) The cells were cotransfected with the PRL promoter and either a wild-type receptor or the P363S mutant TR (20 μg). Reporter activity was measured after 48 h in the presence of 50 nM T3 and/or 1 μM 9-*cis*-RA, as indicated.

activity. These data were obtained with TRα, but similar results were found with the TRβ isoform (data not shown).

To analyze further whether the response to 9-*cis*-RA is mediated by a permissive RXR/TR heterodimer, a heterodimerization-defective mutant TR with P636S, a mutation present in the dimerization interface of the TR viral counterpart v-*erbA* (3), was also used. This mutant TR has been reported to interact with RXR in mammalian two-hybrid experiments (54), but we have observed that in gel retardation assays this mutant TR did not bind the TRE with high affinity in the presence of RXR, although some binding was observed when 9-*cis*-RA was present in the assay (not illustrated). As shown in Fig. 5B, this mutation reduced the response to the retinoid and totally abolished the response to T3 and the synergistic effect of both ligands.

The PRL TRE confers responsiveness to T3 and 9-*cis*-RA to a heterologous promoter. To further prove that the RXR/TR binding site can mediate regulation by ligands of both receptors, fragments of the PRL enhancer containing the TRE alone or the TRE plus the adjacent ERE were cloned in front of the thymidine kinase promoter and transfected into HeLa cells, which express low receptor levels, together with expression vectors for TRα, RXR, or both. Figure 6A shows that, in these cells, expression of TR and/or RXR did not repress transcription but rather caused a ligand-independent stimulation of both reporter plasmids. In addition, incubation with T3 caused a weak increase of promoter activity in TR-expressing cells, whereas 9-*cis*-RA was unable to stimulate this promoter in cells transfected with RXR. However, in cells expressing TR and RXR, 9-*cis*-RA increased reporter activity and was able to cooperate with T3, a finding similar to that observed with the natural promoter in pituitary cells. However, none of the constructs containing fragments of the PRL promoter were stimulated by T3 or the retinoid in nonpituitary HeLa cells (data not shown). As shown in Fig. 6B, the heterologous promoter containing the response element was stimulated by T3 and 9-*cis*-RA in GH4C1 cells, although stimulation was much weaker than that observed with the constructs containing the element in the context of its natural environment in the PRL distal enhancer. In contrast with the results obtained with GH4C1 or HeLa cells, the TRE-containing reporter plasmid was stimulated by T3 but not by 9-*cis*-RA in CV-1 cells, which also express very low receptor levels, after expression of TR and RXR (Fig. 6C). Therefore, in some cell types but not in others, RXR/TR appears to function as a permissive heterodimer, allowing stimulation by 9-*cis*-RA of constructs containing the TRE present in the PRL distal enhancer.

Both RXR and TR can bind ligand and recruit coactivators when bound to the PRL TRE. As stated above, the RXR/TR heterodimer has been considered to be nonpermissive. It is believed that in nonpermissive heterodimers RXR is incapable of ligand binding and therefore acts as a silent partner (15). Although this hypothesis has been recently challenged in a study using a derepression assay system (28), the possibility that 9-*cis*-RA could stimulate PRL gene transcription through a permissive RXR/TR heterodimer requires demonstration that the binding of RXR ligand can cause coactivator recruitment by the DNA-bound receptors. Gel retardation assays illustrated in Fig. 7A show association of the receptor-interacting domains of the coactivators SRC-1 and ACTR fused to

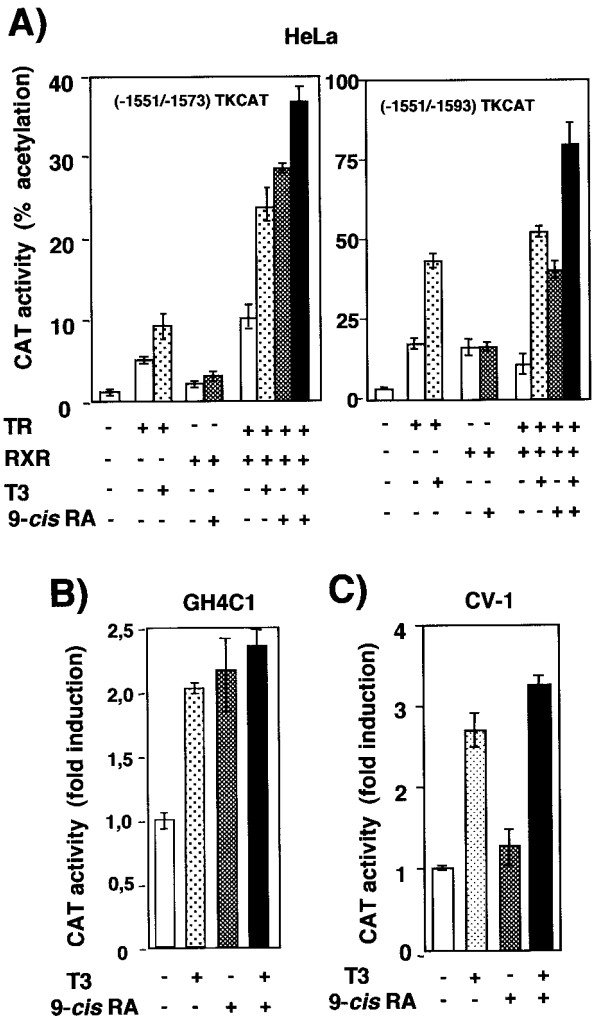

FIG. 6. RXR acts as a silent partner for TR in CV-1 cells but not in GH4C1 or HeLa cells. (A) HeLa cells were transfected with plasmids in which PRL sequences from −1551 to −1573 (which contain the TRE) or −1551 to −1593 (which contain both the TRE and the ERE) were fused to a thymidine kinase (TK)-chloramphenicol acetyltransferase (CAT) reporter gene. The reporter gene was cotransfected with the expression vector for TR (2.5 μg) and/or that for RXR (0.5 μg) as indicated, and luciferase activity was determined after 48 h of incubation with T3 (200 nM) and 9-*cis*-RA (1 μM). (B) Stimulation of the −1551 to −1593 TK-CAT plasmid by the endogenous receptors in GH4C1 cells treated for 48 h with 5 nM T3 and 9-*cis*-RA (1 μM). (C) Reporter activity was determined in CV-1 cells transfected with the −1551 to −1593 TK-CAT plasmid and vectors for TR (200 ng) and RXR (50 ng). Cells were treated as for panel A.

GST upon the binding of T3 and 9-*cis*-RA to the RXR/TR heterodimer bound to the PRL response element. It can be seen that both heterodimeric partners form complexes with the coactivators in the presence of either T3 or 9-*cis*-RA, implying that RXR can bind its ligand even in the context of the DNA-bound heterodimer. Interestingly, there is some coactivator specificity for this response, since 9-*cis*-RA was more efficient in recruiting ACTR than SRC-1 (compare lanes 6 and 12). Furthermore, depending on the ligand used, a slight but consistent change in the mobility of the retarded band containing the ternary complex of the coactivators with RXR/TR was

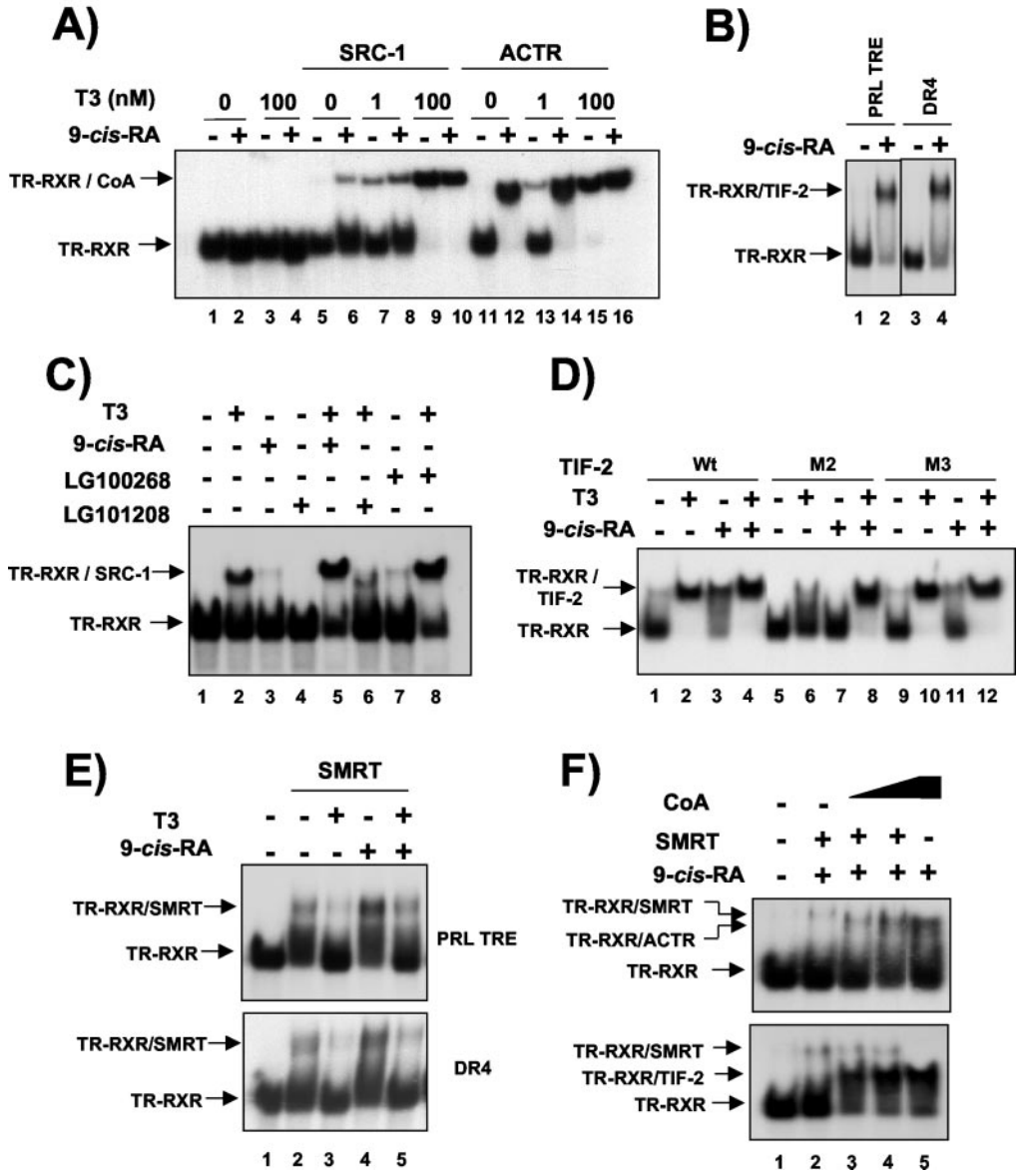

FIG. 7. RXR agonists cause coactivator recruitment by the RXR/TR heterodimer. (A) Gel retardation assays with the PRL TRE oligonucleotide and in vitro-translated TR and RXR. Assays were performed in the presence of the receptor-interacting domains of the p160 coactivators SRC-1 and ACTR fused to GST. In lanes without coactivators (lanes 1 to 4), the same amount of GST alone was added. As indicated, 9-*cis*-RA (1 μM) and T3 (1 and 100 nM) were present in the binding assays. Arrows, mobilities of the heterodimer and the complexes containing the heterodimer and the coactivator (CoA). (B) Gel retardation assays were performed with oligonucleotides conforming to either the PRL TRE or a consensus DR4. The binding of the p160 coactivator TIF-2 to RXR/TR was analyzed in the presence and absence of 1 μM 9-*cis*-RA. (C) Similar assays were performed with SRC-1 in the presence of 100 nM T3 or 1 μM 9-*cis*-RA, the rexinoid LG100268, or the RXR-selective antagonist LG101208. (D) Association of the RXR/TR heterodimer with wild-type His-tagged TIF-2 or with TIF-2 with mutations in the second (M2) or third (M3) LXXLL motif (450 ng). Assays were performed with the PRL TRE and in the presence of 20 nM T3 and 1 μM 9-*cis*-RA as indicated. (E) Gel retardation assays with the receptor-interacting domain of the corepressor SMRT fused to GST, the receptor heterodimer, and either the PRL TRE (top) or DR4 (bottom). Assays were performed with 9-*cis*-RA (1 μM) and/or T3 (20 nM). (F) PRL TRE was incubated with the corepressor in the presence of increasing amounts of GST-ACTR (200 to 600 ng) (top) or His-tagged TIF-2 (300 to 900 ng) (bottom). When indicated, 1 μM 9-*cis*-RA was present in the assays.

detected. This change was better observed when a longer probe was used (data not shown). Figure 7B shows that recruitment of the coactivators by 9-*cis*-RA is not a particular characteristic of the PRL TRE, since this ligand also caused binding of other p160 coactivator, TIF-2, when the heterodimer was bound to a consensus DR4 element. As illustrated in Fig. 7C, the RXR agonist LG100268 was also able to induce binding of a p160 coactivator to the heterodimer (lane 7), whereas LG101208, a RXR-selective antagonist, did not cause coactivator interaction (lane 4). These results again demonstrate that both TR and RXR autonomously bind their ligands and that upon agonist binding each of them can recruit

coactivators. Cooperation of T3 and 9-*cis*-RA in activation of PRL gene expression could result from cooperative coactivator binding. Under conditions in which the retarded heterodimer is not totally supershifted in the assay (Fig. 7C), it could be indeed observed that the combination of 9-*cis*-RA and T3 was more efficient in causing heterodimer-coactivator association than either ligand alone. Furthermore, cooperation of T3 and the RXR agonist was found, whereas the RXR antagonist did not increase, but rather decreased, coactivator recruitment in the presence of T3. Again, slight changes in complex mobility with the different ligands were observed.

The receptor-interacting domain of p160 coactivators is composed of three boxes, each containing the LXXLL motif (19). As shown in Fig. 7D, mutation of box III (M3) had little effect on recruitment of the His-tagged TIF-2 receptor-interacting domain by T3 or 9-*cis*-RA, whereas mutation of box II (M2) strongly decreased the response to T3 and inhibited the response to 9-*cis*-RA. However, the M2 mutant interacted strongly with the heterodimer in the presence of both ligands. Therefore, boxes I and III, which are inefficient, can act synergistically and promote interaction with the receptors when both ligands of the heterodimer are present.

To test the possibility that 9-*cis*-RA could alter corepressor binding to the RXR/TR heterodimer, gel retardation assays were performed with the C-terminal fragment of the corepressor SMRT fused to GST. The amount of corepressor used in the assays to detect binding was high, since heterodimers do not recruit corepressors with the same potency as receptor homodimers (8, 51). As shown in Fig. 7E, a retarded complex was detected in the absence of T3 and, as expected, the hormone caused the disappearance of the retarded band. In contrast, 9-*cis*-RA produced a detectable increase in the intensity of the retarded complex and was able to partially antagonize the effect of T3 on corepressor release. An increase of SMRT binding to the heterodimer in the presence of 9-*cis*-RA was also found with a consensus DR4 (Fig. 7D). To analyze whether under these conditions the RXR/TR heterodimer can still recruit coactivators, assays were performed in the presence of 9-*cis*-RA and SMRT. Figure 7F shows that 9-*cis*-RA caused recruitment of ACTR in the presence of SMRT and that the coactivator inhibited the complex of the heterodimer with the corepressor, which has a slightly lower mobility (top). To better resolve these complexes, additional assays were carried out with SMRT fused to GST and the His-tagged TIF-2 protein. As shown in Fig. 7F (bottom), strong biding of the coactivator to the heterodimer in response to 9-*cis*-RA was found, even in the presence of SMRT.

Role of helix 12 of TR and RXR on coactivator recruitment by T3 and 9-*cis*-RA. To analyze the contribution of the AF-2 domains of each heterodimeric partner to coactivator recruitment by the agonists, gel retardation assays with mutant receptors in which the core AF-2 domain contained in helix 12 was either mutated or deleted were performed. Figure 8A shows the results obtained with the p160 coactivator TIF-2 (top) and with the DRIP205 subunit of the DRIP/TRAP complex (bottom). It was observed that 9-*cis*-RA was also able to induce association of these coactivators with the receptors, more efficiently in the case of TIF-2 (lane 2). Deletion of helix 12 in TR not only abolished the expected recruitment by T3 but also blocked the association of TIF-2 with the heterodimer

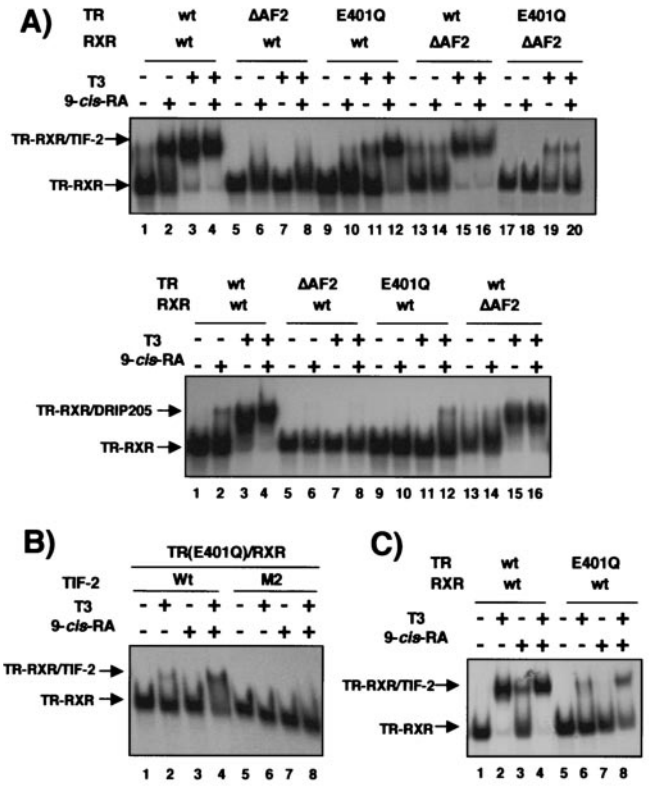

FIG. 8. The TR AF-2 domain is required for coactivator recruitment by the RXR ligand. (A) Gel retardation assays with the receptor-interacting domains of TIF-2 (top) and DRIP205 (bottom) fused to GST and the PRL TRE oligonucleotide. As indicated, wild-type (wt) TR and RXR, receptors lacking helix 12 (ΔAF2), and the point mutant TR TR(E401Q) were used. (B) Assays were performed with TR(E401Q), native RXR, and wild-type (Wt) His-tagged TIF-2 or TIF-2 with a mutation in the second LXXLL box (M2). (C) The consensus DR4 oligonucleotide was incubated with GST–TIF-2 and either wild-type receptors or TR(E401Q). In all panels experiments were performed in the presence and absence of T3 (20 nM) and 9-*cis*-RA (1 μM) as indicated.

in response to 9-*cis*-RA (lanes 5 to 8). Therefore, the TR AF-2 plays a key role in coactivator recruitment by both ligands. The influence of mutation of a conserved glutamic acid residue (E401Q) in helix 12 of TR on the effect of both ligands was also explored. This mutation reduced very significantly association of TIF-2 with the receptors upon incubation with T3 or with 9-*cis*-RA. However, a synergistic effect of both agonists could be observed, and with the combination of 9-*cis*-RA and T3 a strong recruitment of the coactivator to the AF-2-defective heterodimer was found (lanes 9 to 12). This synergistic effect also required the RXR AF-2 domain, since 9-*cis*-RA did not cause coactivator recruitment and was unable to cooperate with T3 in the heterodimer composed of TR with the E401Q mutation [TR(E401Q)] and RXR with AF-2-deleted (lanes 17 to 20). On the other hand, deletion of the RXR AF-2 domain abolished the response to 9-*cis*-RA but allowed TIF-2 recruitment in response to T3 (lanes 13 to 16). Similar qualitative results were obtained with DRIP205, although the effect of 9-*cis*-RA was less marked and TR(E401Q) only weakly recruited the coactivator in the presence of the combination of

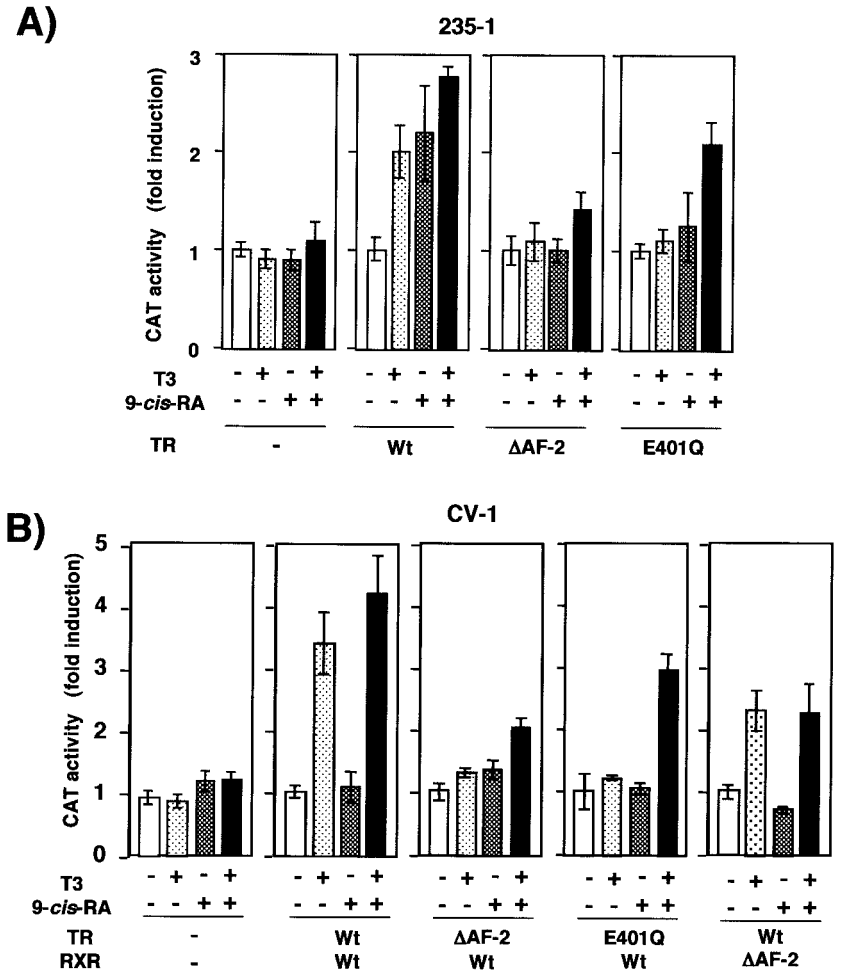

FIG. 9. Transactivation by AF-2 mutant receptors. (A) Pituitary 235-1 cells were transfected with the PRL reporter, which contains the distal enhancer fused to proximal promoter sequences, and expression vectors (15 μg) for wild-type TR, TR(ΔAF-2), TR(E401Q). Luciferase activity was measured after treatment with 50 nM T3 and/or 1 μM 9-*cis*-RA for 48 h and is expressed as the factor by which induction exceeded the value for the untreated control within each group. (B) CV-1 cells were transfected with the PRL TRE fused to the heterologous thymidine kinase (TK) promoter and vectors for native TR (200 ng) and RXR (50 ng) or with the same amount of the specified AF-2 mutant receptors. Chloramphenicol acetyltransferase (CAT) activity was determined after treatment with T3 (5 nM) and/or 9-*cis*-RA (1 μM).

both agonist ligands. As shown in Fig. 8B, the synergistic effect of T3 and 9-cis-RA on TIF-2 recruitment by the AF-2 mutant TR required the integrity of the receptor-interacting domain of the coactivator, as mutation of the second LXXLL motif abolished ligand-dependent association of TIF-2 with the RXR/TR(E401Q) heterodimer. The influence of the helix 12 of TR(E401Q) on coactivator recruitment by both agonists on the consensus DR4 was also tested As shown in Fig. 8C, mutation of the E401 residue also abolished interaction with the coactivator in response to 9-*cis*-RA, and a synergistic effect of both ligands was observed again (lane 8).

Transcriptional regulation by AF-2 mutant receptors. To analyze the effect of the AF-2 mutant receptors on transcriptional regulation by T3 and 9-*cis*-RA, the PRL construct was transfected into 235-1 cells together with expression vectors for wild-type and AF-2 mutant receptors (Fig. 9A). Whereas, as already observed in Fig. 5, both T3 and 9-*cis*-RA increased reporter activity upon expression of the native receptor, deletion of helix 12 of TR abolished the response to both ligands. This is in agreement with the lack of coactivator recruitment by the RXR/TR(ΔAF-2) heterodimer shown in Fig. 8. In addition, mutation of the E401 residue inhibited, as expected, the response to T3, but a significant activation was observed when the hormone was combined with 9-*cis*-RA. This result is also compatible with the in vitro coactivator association with this AF-2-defective heterodimer observed in the presence of both agonists.

The effect of the mutant receptors was also examined in nonpituitary CV-1 cells transfected with the construct containing the PRL TRE fused to the heterologous thymidine kinase promoter. Figure 9B shows again that in this cell type expression of native TR conferred regulation by T3, but not by 9-*cis*-RA. Additionally, no response to T3 was observed upon expression of the AF-2-defective mutant TRs. However, confirming that the lack of transcriptional activity of the E401 mutant TR can be reversed when both heterodimeric partners are occupied, a significant activation in the presence of T3 plus 9-*cis*-RA was observed in cells transfected with this mutant TR.

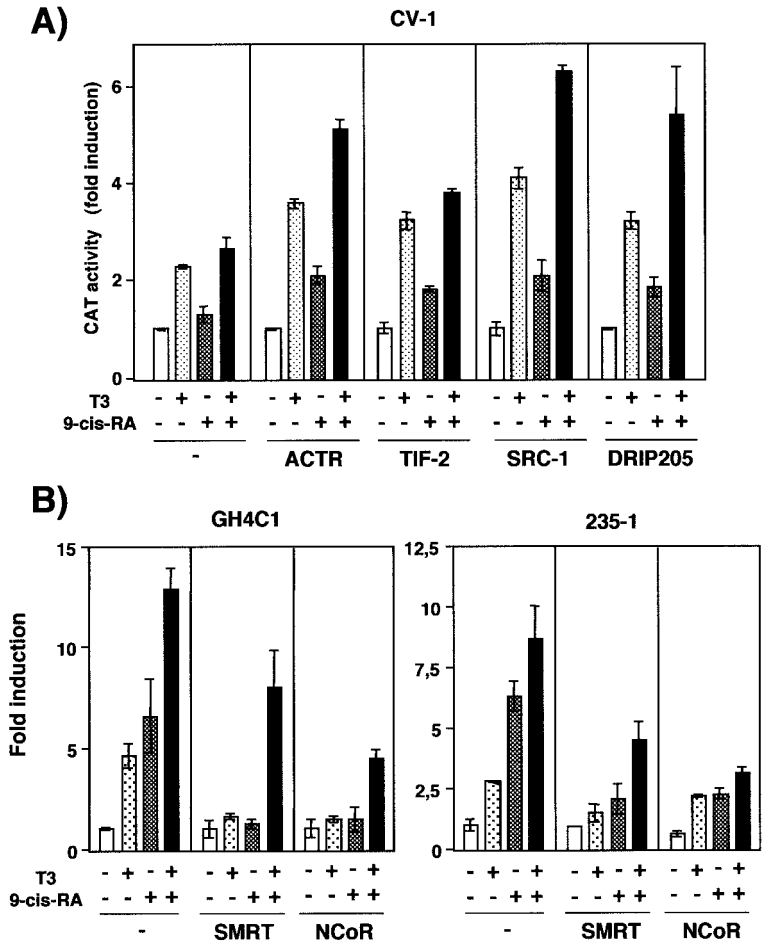

FIG. 10. Expression of coregulators modulates responsiveness to 9-*cis*-RA. (A) CV-1 cell were transfected with the thymidine kinase (TK)-chloramphenicol acetyltransferase (CAT) construct containing the PRL TRE and expression vectors for TR (200 ng) and RXR (50 ng). Vectors (100 ng) for the coactivators ACTR, TIF-2, SRC-1, and DRIP205 or a noncoding vector (100 ng) was cotransfected with the reporter and the receptors, and CAT activity was determined after treatment with T3 (5 nM) and/or 9-*cis*-RA (1 μM) for 48 h. Data are expressed as the factors by which induction exceeded the value obtained for the corresponding control untreated cells. (B) Pituitary GH4C1 cells were cotransfected with the PRL promoter construct and 200 ng of expression vectors for SMRT or NcoR. Luciferase activity was determined after incubation with 5 nM T3 and/or 1 μM 9-*cis*-RA. In 235-1 cells the PRL reporter was cotransfected with 15 μg of TR and 500 ng of SMRT or NcoR.

In agreement with the finding that the RXR AF-2 domain does not play a major role in in vitro recruitment with coactivators in response to T3, expression of RXR lacking helix 12 allowed stimulation by T3, although stimulation was somewhat weaker than that found upon expression of native RXR.

Coactivator and corepressor availability modulates the response to 9-*cis*-RA. The different transcriptional responses of pituitary and CV-1 cells to 9-*cis*-RA could be due to different availabilities of coregulators. To test this possibility, the construct containing the PRL TRE was cotransfected into CV-1 cells with expression vectors for the p160 coactivators ACTR, TIF-2, and SRC-1, as well as for DRIP205. As shown in Fig. 10A, expression of the coactivators increased the response to T3 and, more importantly, allowed stimulation by 9-*cis*-RA. In contrast, expression of coactivators was unable to increase the response of the PRL promoter to either T3 or 9-*cis*-RA in pituitary GH4C1 or 235-1 cells (not illustrated), showing that in these cells the amount of endogenous coactivators is sufficient to elicit a maximal transcriptional response.

To analyze whether the availability of corepressors could also modulate the response to 9-*cis*-RA in cells in which this ligand promotes transactivation, the PRL construct was cotransfected into GH4C1 and 235-1 cells together with vectors for the corepressors SMRT and NcoR. Figure 10B shows that expression of both corepressors reduced the response to T3 and 9-*cis*-RA in the pituitary cells. However, when both ligands were combined, a synergistic effect was still observed and significant reporter stimulation was found. Interestingly, transfection of amounts of corepressors higher than those used in Fig. 9B did not further reduce these responses but rather allowed a stronger ligand-dependent stimulation (not illustrated).

DISCUSSION

It has been previously found that lactotroph tumor cells respond to T3 with either an increase or a decrease in PRL gene expression (11, 44). In this work we have shown that in the rat GH4C1 somatolactotroph cell line T3 increases PRL

transcripts as well as PRL promoter activity in transient-transfection studies. Most likely this represents a physiological action of the hormone, since we have also observed a profound decrease of PRL mRNA levels in pituitaries of thyroidectomized rats that is reversed upon thyroid hormone treatment (G. Bedó and A. Aranda, unpublished observations). This regulation contrasts with the observation that hypothyroidism in human patients is often associated with an increase in serum PRL levels (48). Although this is possibly due to central effects, it has been shown that T3 represses the activity of the human PRL promoter. The human promoter appears to contain both a positive and a negative TRE, and the negative effect is stronger and could involve cross talk between the thyroid hormone receptor and AP-1 (38). This demonstrates the existence of species specificity in regulation of PRL gene expression.

We also show in this work that 9-*cis*-RA increases the levels of PRL transcripts and stimulates the activity of reporter plasmids containing the PRL enhancer in GH4C1 cells. A common element in the enhancer region, the rat PRL TRE, mediates regulation by both T3 and the RXR agonist. As in many TREs, the hemisites of the PRL TRE are arranged as DRs separated by 4 nucleotides (1, 53). As expected from this configuration, the PRL TRE binds RXR/TR heterodimers with high affinity but shows little affinity for TR or RXR homodimers or monomers. The PRL TRE is adjacent to the ERE, but it functions as a separate element, as mutation of the TRE abolishes stimulation by both T3 and the 9-*cis*-RA but does not affect activation by estradiol.

The TRE functions as a bona fide response element, since it can confer ligand responsiveness to a heterologous promoter both in pituitary and nonpituitary cells. The finding that the element fused to the heterologous promoter is weakly stimulated by T3 and 9-*cis*-RA in pituitary cells, in comparison with the strong stimulation of the natural PRL sequences, suggests that the promoter context is important in determining the functional strength of the response element. On the other hand, the PRL constructs which contain the TRE were not activated by these ligands in nonpituitary cells. The lack of functionality of the PRL TRE in these cells could be related to the absence of the pituitary-specific factor GHF-1/Pit-1, which binds to the PRL gene and which is needed for ligand-dependent transcription of the PRL and growth hormone (GH) genes by other nuclear receptors (5, 12, 34, 36, 45, 46). The requirement of GHF-1/Pit-1 for ligand-dependent stimulation of PRL gene transcription is also deduced from the finding that a construct extending to nucleotide −1597 that contains the PRL TRE but that lacks the distal enhancer binding sites for the pituitary factor is not activated by T3 or 9-*cis*-RA in GH4C1 cells (A. I. Castillo and A. Aranda, unpublished observations).

It was normally assumed that in TR signaling the only function of RXR was to facilitate the binding of TR to the TRE. This was based on evidence obtained by Forman et al. (15) showing that ligand binding by RXR was abolished when this receptor heterodimerizes with TR. However, our results do not support this model, in which RXR ligand binding is possible only when TR is occupied by the hormone, and rather show that RXR does not act as a silent partner for TR in stimulation of PRL gene expression. In fact, a main finding of this work is the demonstration that RXR/TR can act as a permissive heterodimer, allowing stimulation of transcription by the ligands of both subunits of the heterodimer. The use of a RAR-selective ligand, together with the finding that RXR/RAR heterodimers do not associate with this element, dismisses the possibility that 9-*cis*-RA could stimulate PRL transcription through binding to RAR. Furthermore, the RXR-selective ligand LG100268 was able to stimulate PRL transactivation, although with less potency than the natural RXR agonist. The rexinoid, which acts as an agonist for RXR homodimers (26), presents some dissimilarities from the natural agonist 9-*cis*-RA which could explain this difference. The crystal structure of RXR bound to this retinoid has shown that the C-terminal helix 12, which contains the receptor AF-2 domain, is trapped in a novel position not seen in other liganded nuclear receptors (30). Furthermore, LG100268 is unable to release corepressors from RXR unless coactivators are present (30).

Stimulation by 9-*cis*-RA was found in pituitary 235-1 cells transfected with the PRL distal enhancer upon expression of TR. Furthermore, the effect of the retinoid was decreased in cells expressing a heterodimerization-defective mutant TR. Also, in HeLa cells transfected with the PRL TRE fused to a heterologous promoter, expression of the RXR/TR heterodimer conferred responsiveness to both T3 and 9-*cis*-RA. These results clearly prove that a permissive RXR/TR heterodimer can support transcription by agonists of both receptors under appropriate circumstances. However, whereas a response to T3 was found in pituitary and HeLa cells, stimulation of the TRE-containing promoter by 9-*cis*-RA was not observed in CV-1 cells. This shows that the cellular context can be crucial in determining whether a given element can confer regulation by one or both ligands of the receptor heterodimer.

A common element in the 5′-flanking region of the rat GH gene mediates regulation by both T3 and retinoids (4, 17), and an RXR-selective ligand has been used to demonstrate that RXR activates the rat GH promoter in pituitary cells through the TRE (10). In the light of our present results, and since RXR does not bind as a heterodimer to this element of the GH promoter (35), it is likely that the liganded RXR could also function as a partner for TR and that a permissive heterodimer could be responsible for stimulation of the pituitary GH gene by the rexinoid. Permissiveness of RXR/TR heterodimers for PRL and GH gene expression could be due to the promoter context or to selective interaction with other factors that bind these promoters. However, this does not appear to be the case, because the PRL TRE confers T3 and 9-*cis*-RA responsiveness to a heterologous promoter. Moreover, we have found cooperation of T3 and all-*trans*-RA in stimulation of reporter plasmids containing consensus TREs in pituitary cells (17). Therefore, somatolactotrophs could present a cellular environment particularly advantageous to overcome RXR subordination within heterodimers with TR.

We have been able to demonstrate that, in the RXR/TR heterodimer bound to the PRL TRE, each partner can independently bind ligand and recruit coactivators. Similar results have been recently shown for the RXR/RAR heterodimer (18), previously considered to be unable to bind RXR ligand when RAR was unoccupied. The RXR ligand could elicit interaction of coactivators with this receptor or could induce a conformational change in TR, which then would recruit the coactivator. Our observation that deletion of the RXR AF-2

domain abolishes association of coactivators in response to 9-*cis*-RA suggests that a coactivator molecule binds directly to the RXR moiety. Furthermore, we have observed that the conformation of the coactivator-heterodimer complexes formed is different depending of the bound ligand, as their mobilities in gel retardation assays differ. Therefore, our data are compatible with a model in which each subunit of the heterodimer creates a surface for coactivator interaction. This is in agreement with recent data indicating that a single coactivator molecule binds to receptor heterodimers. The receptor-interacting domains of the coactivators contain three copies of the signature motif LXXLL (19), and each partner of the heterodimer appears to recognize a different LXXLL box of the coactivator (13, 49). In the presence of both ligands synergy would originate from the cooperative binding of the two receptor-interacting motifs (18). Our results have shown that the second LXXLL box is required for the binding of the coactivator TIF-2 to the heterodimer in response to T3 and 9-*cis*-RA. However, in the absence of box II, both ligands act synergistically and the weak I and III motifs are sufficient for efficient binding to the receptors.

Although each receptor in the heterodimer can autonomously bind coactivators, our results also demonstrate that there is an important cross talk between the receptor partners. For instance, deletion of the TR AF-2 core domain abolishes coactivator recruitment not only in response to T3 but also in response to 9-*cis*-RA, and this is translated into loss of the transcriptional response to both compounds in cells expressing the truncated receptor. This is reminiscent of the ligand phantom effect observed with other heterodimers such as LXR/RXR, in which the activation potential of LXR is enabled by ligand binding to its partner (50) or by the binding of a synthetic ligand to RXR that mimics the effects observed when the hormone is bound to RAR (43).

An interesting novel observation was obtained with a TR with a mutated conserved residue that is involved in association with the LXXLL box (13) and that is required for ligand-dependent transcriptional activation (2). We have shown that the heterodimer of RXR with TR(E401Q) displays a strongly reduced ability to bind coactivators in response to T3, but binding is significantly restored when RXR is occupied. Remarkably, the mutated receptor is transcriptionally inactive in cells incubated with T3 alone but can stimulate transcription when the hormone is combined with 9-*cis*-RA. Since point mutations of the TRβ isoform in helix 12 are present in some patients with thyroid hormone resistance syndrome (9), our results open the interesting possibility that some transcriptional activity of AF-2-deficient receptors could be found under conditions in which the RXR ligand is present. From our data it can be also inferred that the effects of 9-*cis*-RA on TRE-dependent transactivation should depend on the cellular pattern of coactivator expression. We have shown that there is some specificity in coactivator binding and that some coactivators are more efficiently recruited than others in response to 9-*cis*-RA. Therefore, it can be expected that rexinoid responses through an RXR/TR heterodimer should be selectively found in those cell types expressing a favorable combination of coactivators and that RXR could be silent in other cellular contexts.

The cellular levels of corepressors could also play an important role in the transcriptional response mediated by the agonists of the RXR/TR heterodimer. For RXR/RAR, rexinoids induce coactivator recruitment to RXR but cannot dissociate corepressors (18). Since coactivators and corepressors have similar interaction surfaces in the receptor (23, 33, 37), binding of both is mutually exclusive. It has been proposed that RXR ligands can transactivate only when the heterodimeric partner interacts weakly with corepressors or when coactivator expression in a particular cell dominates corepressor content (18). In agreement with this hypothesis we have observed that overexpression of corepressors in pituitary cells inhibits PRL promoter transactivation not only by 9-*cis*-RA but also by T3. Interestingly, a synergistic effect of both ligands is still observed even in the presence of high corepressor levels. These data, as well as our results showing that expression of coactivators allows the response to 9-*cis*-RA in CV-1 cells, underline the importance of the levels of these coregulators for RXR to act as a nonsilent partner of TR.

For RXR/TR it has been recently proposed that the binding of the RXR ligand could induce dissociation of corepressors from TR and thus may serve to modulate TR activity (28). However, our data do not support this model, since incubation with 9-*cis*-RA increases the binding of the corepressor SMRT to the heterodimer in gel retardation assays using both the PRL TRE and a consensus DR4. Since unliganded RXR does not bind corepressors because its helix 12 masks the corepressor binding surface (52), these results again suggest that the binding of ligand to RXR results in a linked conformational change in TR. However, it is also possible that RXR could bind corepressors upon ligand binding. The increase in corepressor recruitment by 9-*cis*-RA is in apparent contradiction with the stimulation of gene expression seen in the functional assays. However, we have observed that the retinoid causes strong recruitment of coactivators even in the presence of corepressors, and under these conditions stimulation would be favored. In any case, the enhancement in corepressor recruitment by 9-*cis*-RA could also contribute to the silencing of the transcriptional response mediated by this ligand, particularly in cells with high corepressor content or with a high corepressor-to-coactivator ratio.

In summary, our findings indicate that RXR ligands can elicit PRL gene transcription through a permissive heterodimer with TR. This demonstrates an unexpected role for RXR in TR signaling and argues against a silent-partner model for RXR. Therefore, future studies are needed to analyze the function of RXR/TR heterodimers and to reevaluate the actions of both receptors and agonist and antagonist ligands in different genes and cell types.

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THE RETINOID X RECEPTOR LIGAND RESTORES DEFECTIVE SIGNALLING BY THE VITAMIN D RECEPTOR

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Introducción al Capítulo 2

Como hemos visto en el capítulo 1 de esta tesis, el RXR es capaz de unir su ligando y estimular la transcripción de un gen natural demostrando una clara permisividad del heterodímero TR/RXR a la acción de los rexinoides. A tenor de esas observaciones nos planteamos revisar el papel del RXR en el heterodímero VDR/RXR, el único de los heterodímeros del RXR que aún podría considerarse como "no permisivo". Tradicionalmente se atribuía al RXR la única función de facilitar la unión y reconocimiento de los elementos de respuesta por estos heterodímeros. A lo largo de este estudio mostramos cómo el RXR ejerce un papel activo dentro del heterodímero VDR/RXR más allá del de incrementar la afinidad por el DNA. Hemos visto cómo la unión del 9-*cis*RA es capaz de provocar reclutamiento de coactivadores por el heterodímero y de estimular la actividad transcripcional dependiente de VitD ensayada mediante experimentos de transfección transitoria o mediante el análisis de los niveles de RNA mensajero del gen *cyp*24. Además el ligando del RXR coopera con la VitD produciendo una clara aditividad de acción. Mas aún, la combinación con el rexinoide permite recuperar la respuesta transcripcional del heterodímero en situaciones defectivas como es el caso de receptores o coactivadores mutados y de ligandos poco activos. Por último, el 9-*cis*RA es capaz de cooperar con dosis bajas de VitD o del antagonista parcial ZK155222 para promover la diferenciación de células de cáncer de colon SW480ADH estimulando la expresión de E-cadherina. Todos estos hallazgos nos muestran un claro papel del ligando del RXR en las respuestas fisiológicas complejas mediadas por VDR y descartan la antigua idea de la existencia de heterodímeros del RXR en los cuales éste actúe como "un compañero silencioso" de su pareja heterodimérica. El RXR siempre tiene algo que decir.

La alumna ha contribuido a este trabajo tanto en el diseño experimental como en la discusión y juicio crítico sobre los resultados que lo componen. Desde un punto de vista técnico ha intervenido en la realización de ensayos EMSA, transfecciones, técnicas de siRNA y Western Blot, así como en la mutagénesis de los receptores VDR y RXR y en la caracterización funcional de los mismos.

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The retinoid X receptor ligand restores defective signalling by the vitamin D receptor

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It is assumed that the retinoid X receptor (RXR) acts as a silent partner to the vitamin D receptor (VDR) with its only function to increase affinity of VDR/RXR to its DNA recognition site. In this study, we show that the RXR ligand 9-*cis*-retinoic acid (9-*cis*-RA) induces recruitment of coactivators by the DNA-bound heterodimer and potentiates vitamin D-dependent transcriptional responses. The presence of 9-*cis*-RA increases induction of *cyp24* transcripts and differentiation of colon cancer cells by vitamin D, confers significant agonistic activity to a VDR ligand with very low agonistic activity and can even restore transcriptional activity of an AF-2 mutant VDR that causes hereditary rickets. This study shows that, in VDR/RXR heterodimers, allosteric communication triggered by the RXR ligand has a previously unrecognized role in vitamin D signalling, with important physiological and therapeutic implications.

Keywords: vitamin D receptor; retinoid X receptor; heterodimer; coactivators

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INTRODUCTION

Vitamin D (1α,25-dihydroxyvitamin D3, calcitriol) has a key role in calcium homeostasis, has suppressive effects on the immune system, promotes cell differentiation and inhibits proliferation of transformed cells. This has led to the search of vitamin D analogues with therapeutic effects but without calcemic activity. Their actions are mediated by binding to the nuclear vitamin D receptor (VDR) that regulates gene expression by binding to vitamin D responsive elements (VDREs), which are typically composed of a direct repeat of the sequence PuGGT/GTCA spaced by three nucleotides (DR3; Aranda & Pascual, 2001).

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Stimulation of transcription by nuclear receptors is mediated through recruitment of coactivators. Three copies of the LXXLL motif, where X denotes any amino acid, contained in the receptor-interacting domain of the p160 coactivators, mediate association with the receptors (Heery *et al*, 1997). This motif is also present in the DRIP205 subunit of TRAP/DRIP (thyroid receptor-associated protein/vitamin D receptor interacting protein), also known as the Mediator complex. Ligand binding triggers a conformational change in the receptor that creates a surface for coactivator binding. Repositioning of the ligand-dependent transcriptional activation function (AF-2) located at the carboxy-terminal helix 12 (H12) of the ligand-binding domain (LBD), together with residues in H3, H4 and H5, forms a hydrophobic groove that accommodates this motif (Gronemeyer *et al*, 2004). A conserved glutamic acid in H12 (E420) and an invariable lysine (K246) in H3 of the VDR contact directly with the LXXLL motif and form a charge clamp that stabilizes binding (Vanhooke *et al*, 2004). Accordingly, mutations in these residues render a VDR unable to mediate vitamin D-dependent transactivation (Jimenez-Lara & Aranda, 1999).

Heterodimerization of VDR, as well as other nuclear receptors, with the retinoid X receptors (RXRs) increases DNA binding and transcriptional activity (Gronemeyer *et al*, 2004). Permissive heterodimers can be independently activated by an RXR ligand (9-*cis*-retinoic acid (9-*cis*-RA)), by an agonist of its partner receptor or by both ligands in a synergistic manner. However, in nonpermissive heterodimers, the ligand-induced transcriptional activities of RXR are suppressed, and it was even proposed that formation of the heterodimer could preclude binding of the ligand to RXR, although this hypothesis has been recently challenged (Germain *et al*, 2002; Castillo *et al*, 2004). Some heterodimers show conditional permissivity. In conditional heterodimers, such as retinoic acid receptor (RAR)/RXR, a full response occurs only in the presence of an RXR ligand. Statistical coupling analysis led to the identification of a network of residues in the LBD crucial for allosteric function, as their mutation can convert a permissive heterodimer into a conditional one and even discriminate between different ligands of a given receptor (Shulman *et al*, 2004). In this analysis, the VDR/RXR heterodimer has been considered to be a strict nonpermissive heterodimer, which cannot be activated by the RXR ligand either in the presence

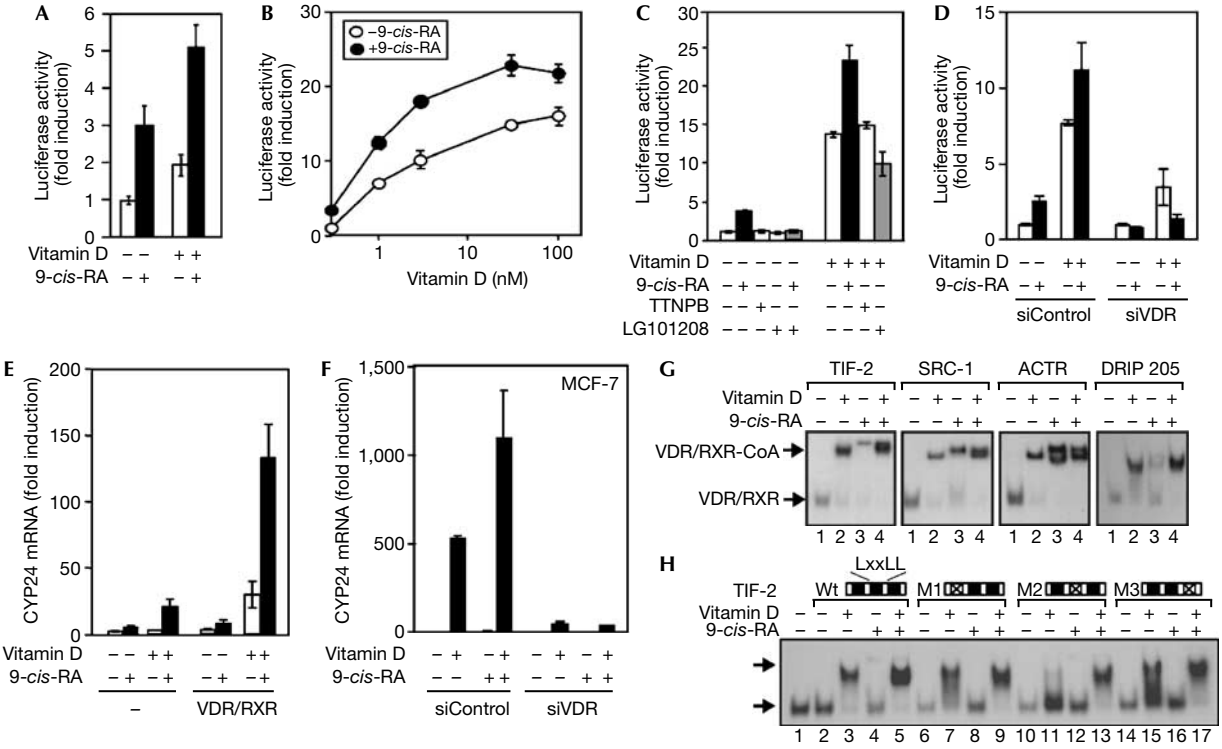

Fig 1 | The retinoid X receptor ligand stimulates transcriptional activity and coactivator recruitment by vitamin D receptor/retinoid X receptor. (**A**) 293-T cells were transfected with the 4xVDRE reporter plasmid and incubated with vitamin D (3 nM) and/or 9-*cis*-RA. In (**B**), the plasmid was co-transfected with VDR and RXR and the cells were incubated with 9-*cis*-RA alone or in combination with the indicated concentrations of vitamin D. (**C**) Cells were transfected with the heterodimer and treated with vitamin D (3 nM) and/or 9-*cis*-RA, TTNPB or LG101208. (**D**) Reporter activity determined in cells transfected with the receptors in the presence of control siRNA (siControl) or VDR siRNA (siVDR). (**E**) 293-T cells were transfected with the heterodimer or an empty vector and the levels of *cyp24* transcripts were determined in cells treated for 4 h with vitamin D and/or 9-*cis*-RA. (**F**) *cyp24* transcripts in MCF-7 cells treated for 12 h with the ligands. (**G**) Electrophoretic mobility-shift assays with *in vitro*-translated VDR and RXR and the p160 coactivators (TIF-2, SRC-1, ACTR), or DRIP205 fused to glutathione *S*-transferase. Mobilities of heterodimers and complexes with the coactivator (CoA) are shown by arrows. (**H**) Association of VDR/RXR with wild-type His-tagged TIF-2 or with mutants in the first (M1), second (M2) or third (M3) LXXLL motifs. 9-*cis*-RA, 9-*cis*-retinoic acid; RXR, retinoid X receptor; siRNA, short interfering RNA; VDR, vitamin D receptor.

or absence of the VDR agonist, although an allosteric modification of unliganded RXR by liganded VDR has been proposed (Bettoun *et al*, 2003).

In this study, we have re-examined the role of the RXR ligand in transcription by VDR/RXR. We find that this heterodimer can recruit coactivators in response to either vitamin D or 9-*cis*-RA, and that both cooperate to stimulate the activity of VDRE reporters to increase transcription of the *cyp24* gene or to promote differentiation of colon carcinoma cells. Furthermore, binding of the RXR agonist causes association with coactivators and transcriptional stimulation by a VDR ligand previously defined as a partial antagonist or even by an inactive AF-2 VDR mutant. These results show that the RXR agonist has a previously unrecognized role in signalling by VDR, with important physiological and pharmacological implications.

RESULTS AND DISCUSSION

VDR/RXR can act as a conditional heterodimer

It is assumed that the RXR ligand cannot autonomously induce transcription from VDR/RXR. However, in 293-T cells transfected with a 4xVDRE reporter, 9-*cis*-RA caused a consistent increase (about threefold) of reporter activity and was able to cooperate with vitamin D, which in these cells caused a weak stimulation (Fig 1A). On expression of the heterodimer, vitamin D caused a strong dose-dependent transactivation, and 9-*cis*-RA caused a further induction (Fig 1B). Similar results were obtained with constructs containing single VDREs (DR3T, DR3G and IP9), or with the VDRE-containing *cyp24* promoter (supplementary Fig 1 online).

In contrast to 9-*cis*-RA, the RAR-specific ligand TTNPB was unable to induce basal activity or to cooperate with vitamin D, excluding the possibility that activation by 9-*cis*-RA could be mediated by cryptic binding of RAR/RXR (Fig 1C). In addition, the RXR antagonist LG101208 abolished induction by 9-*cis*-RA and slightly decreased transactivation by vitamin D, showing that binding to RXR mediates the effect of the retinoid. Finally, short interfering RNA (siRNA) knockdown of VDR not only affected the response to vitamin D but also abolished induction by 9-*cis*-RA and the cooperation of both ligands (Fig 1D), showing that the action of 9-*cis*-RA was mediated by VDR/RXR. The same was observed with the *cyp24* reporter (supplementary Fig 2A online).

The RXR agonist also induced transcription from an endogenous gene. *cyp24* transcripts were induced synergistically by

9-*cis*-RA and vitamin D in 293-T cells (Fig 1E). The effect of vitamin D was weaker than that of 9-*cis*-RA in cells expressing endogenous receptors. However, this situation was reversed on expression of VDR/RXR, when the action of vitamin D was strongly enhanced and the effect of the RXR ligand was only weakly induced. The synergistic effect of both ligands to induce *cyp24* gene transcription was also observed in MCF-7 cells, in which endogenous receptors mediated a strong increase by vitamin D that was further induced by 9-*cis*-RA. This effect was also abolished after knockdown of VDR by siRNA (Fig 1F), showing that VDR/RXR mediates induction of *cyp24* transcripts by these ligands. These results show that the VDR/RXR heterodimer cannot be considered as nonpermissive and that it rather behaves as a conditionally permissive heterodimer.

Activation by 9-*cis*-RA suggests that its binding to RXR can result in recruitment of coactivators by the DNA-bound VDR/RXR. Fig 1G shows that both 9-*cis*-RA and vitamin D cause association of p160 and DRIP205 coactivators with the receptors, demonstrating that dimerization with VDR does not preclude binding of ligand to RXR. Similar results were obtained with the heterodimers of RXR with RAR (Germain *et al*, 2002) or the thyroid hormone receptor (Castillo *et al*, 2004). The ternary complex of the coactivators with the heterodimer had a slower mobility with 9-*cis*-RA than vitamin D, showing that different conformations of the complex were obtained depending on the ligand. In general, recruitment was enhanced when both ligands were combined, suggesting that they cooperate to recruit the coactivator. This was also observed with various natural VDREs (supplementary Fig 3 online). The use of a mutant coactivator allows a clear detection of the effect of the RXR ligand. Mutation of LXXLL motifs I and III (M1 and M3) had little effect, whereas mutation of motif II (M2) essentially abolished recruitment of transcriptional intermediary factor 2 (TIF-2) by vitamin D. However, both ligands acted synergistically and promoted a strong interaction with the M2 mutant (Fig 1H). These results show that RXR does not act as a silent partner for VDR with the only function of increasing binding to the VDRE.

Role of RXR ligand in coactivators recruitment

In Fig 2, binding of p160 coactivators was analysed in response to 9-*cis*-RA, vitamin D and the vitamin D analogue ZK159222. This compound binds to VDR with high affinity but has very low agonistic activity, antagonizing transactivation by vitamin D (Herdick *et al*, 2000; Ochiai *et al*, 2005). In contrast to vitamin D (lane 3), ZK159222 was unable to cause coactivators recruitment (lane 5). However, robust binding was found when this ligand was combined with 9-*cis*-RA (lane 6). Modelling of the structure of VDR bound to ZK159222 shows that its side chain displaces H12, preventing normal association with coactivators (Tocchini-Valentini *et al*, 2004). Our finding that 9-*cis*-RA restores this association suggests that binding of ligand to RXR changes the conformation of its partner, repositioning H12 to an agonist location.

Electrophoretic mobility-shift assays (EMSAs) were also performed with receptors lacking H12 (ΔAF2). Deletion of H12 in RXR did not affect vitamin D-dependent coactivator binding, but had a paradoxical effect on the action of ZK159222, which was able to induce recruitment even in the absence of 9-*cis*-RA (lane 11), although synergism was not observed (lane 12). Deletion of

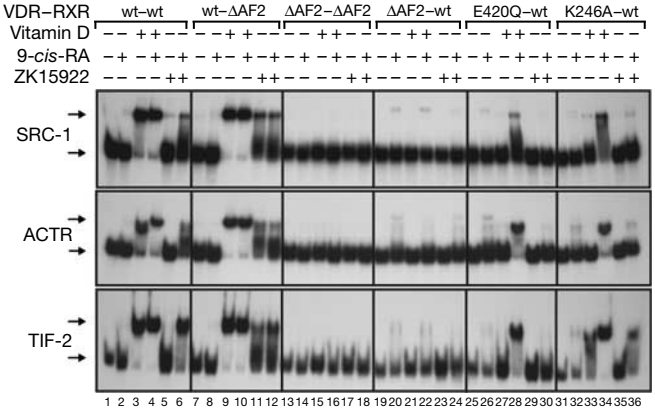

Fig 2 | Recruitment of coactivators by vitamin D receptor mutants and ZK159222 in the presence of 9-*cis*-retinoic acid. Electrophoretic mobility-shift assays were performed with SRC-1, ACTR and TIF-2. As indicated, wild-type (wt) VDR and RXR, receptors lacking H12 (ΔAF-2) and the mutants VDR E420Q and K246A were used. Experiments were performed in the presence and absence of the indicated ligands. 9-*cis*-RA, 9-*cis*-retinoic acid; RXR, retinoid X receptor; VDR, vitamin D receptor.

the AF-2 domains of both receptors totally abolished coactivator recruitment (lanes 13–18), and deletion of VDR AF-2 allowed recruitment by 9-*cis*-RA, but blocked coactivators association by vitamin D, as well as the synergism between the RXR ligand and ZK159222 (lanes 19–24). The inactive VDR mutants E420Q and K246A were also used. Remarkably, the E420 mutation has been detected in families with hereditary vitamin D-resistant rickets without alopecia (Malloy *et al*, 2002). As expected, mutation E420Q inhibited recruitment by vitamin D (lane 27). However, with the combination of 9-*cis*-RA and vitamin D, a strong association of the coactivator to the defective heterodimer was found (lane 28). This synergism was not observed when 9-*cis*-RA and ZK159222 were combined (lane 30). Similar results were obtained with the H3 mutant K264A, as vitamin D alone did not recruit coactivators, but robust binding was found when both ligands were combined (lane 34). These results indicate that the RXR ligand alters the structure of the heterodimer, allowing formation of productive coactivator binding surfaces even when residues that interact with the LXXLL motif are mutated. To gain further insight into the structural requirements for coactivators recruitment by the heterodimer, point mutants on the coactivator binding surface of both receptors were used (supplementary Fig 4 online). Mutations in H3 and H4 of VDR affected binding by vitamin D but did not reduce the response to 9-*cis*-RA, whereas equivalent mutations in RXR were able to abolish recruitment by this ligand. Furthermore, point mutations in RXR H12 had a stronger effect than deletion of this helix—they not only inhibited the response to 9-*cis*-RA, but also markedly reduced binding of coactivators by vitamin D.

RXR agonist restores activation by a mutant VDR

The finding that the RXR ligand causes coactivator recruitment by an inactive AF-2 VDR mutant suggests that transcriptional activity of this receptor could be restored in the presence of 9-*cis*-RA. This is indeed illustrated in Fig 3. Vitamin D strongly increased reporter

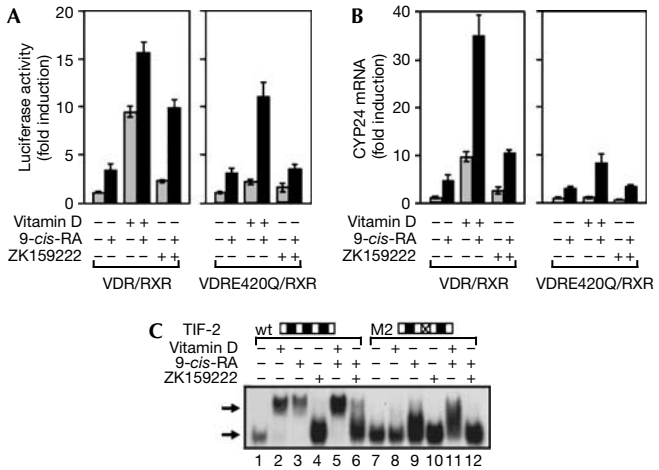

Fig 3 | 9-*cis*-retinoic acid restores the transcriptional activity of mutant vitamin D receptor/retinoid X receptor heterodimers and confers agonistic activity to ZK159222. (**A**) Reporter activity in 293-T cells expressing wild-type VDR/RXR or a heterodimer of RXR with the E420Q VDR mutant. (**B**) *cyp24* messenger RNA levels measured in the same groups after 4 h incubation with vitamin D, ZK159222 and/or 9-*cis*-RA, as indicated. (**C**) Electrophoretic mobility-shift assays with the native VDR/RXR heterodimer and either wild-type (wt) TIF-2 or the mutant in the second LXXLL motif (M2). 9-*cis*-RA, 9-*cis*-retinoic acid; RXR, retinoid X receptor; VDR, vitamin D receptor.

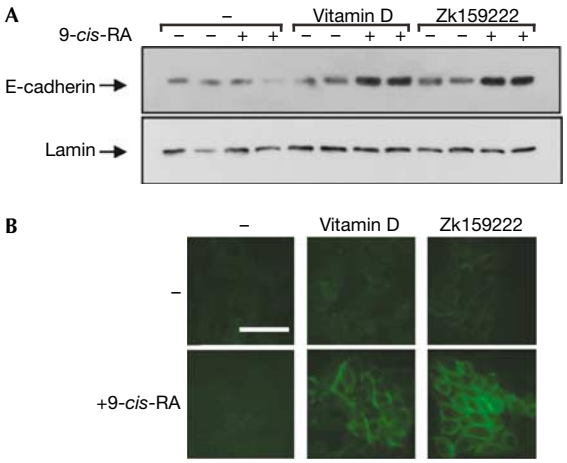

Fig 4 | Cooperation of vitamin D receptor and retinoid X receptor ligands on differentiation of colon carcinoma cells. (**A**) E-cadherin levels in SW480-ADH cells treated with the VDR ligands in the presence and absence of 1 μM 9-*cis*-RA. The membrane was reprobed with an anti-lamin antibody. (**B**) After the same treatments, differentiation was tested by immunofluorescence with E-cadherin. Scale bar, 100 μm. 9-*cis*-RA, 9-*cis*-retinoic acid. RXR, retinoid X receptor; VDR, vitamin D receptor.

activity on expression of the native receptor, whereas the E420 mutation prevented the ligand-dependent response. However, strong transactivation by the AF-2 mutant was observed when vitamin D was combined with 9-*cis*-RA (Fig 3A). A marked synergistic effect of both ligands on endogenous *cyp24* messenger RNA induction was also obtained. Vitamin D alone was essentially inactive to augment *cyp24* transcripts in cells expressing the E420Q mutant, whereas a significant increase was found in the presence of both ligands (Fig 3B). These results are compatible with the '*in vitro*' coactivator association to this AF-2-defective heterodimer observed in the presence of both agonists, and suggest that the use of rexinoids could have a therapeutic effect in patients with the E420 point mutation. In addition, the fact that these patients do not present with alopecia could be due to residual transcriptional activity; it could also be a consequence of activation of the heterodimer by endogenous RXR ligands, the concentration of which could be locally regulated.

Recruitment of coactivators by ZK159222 in the presence of 9-*cis*-RA also suggested that this compound could have transcriptional activity under these conditions. Fig 3A shows that the VDRE reporter was indeed synergistically stimulated by these ligands in cells expressing native receptors. This synergism was also observed when *cyp24* transcripts were quantified (Fig 3B). These results show the importance of the occupancy of RXR on VDR signalling, as the presence of the RXR ligand can convert ZK159222 into a rather strong agonist. Interestingly, the response obtained with the combination of ZK159222 and 9-*cis*-RA was at least as strong as that observed with vitamin D alone in the absence of the RXR ligand. The cooperation between both ligands could have important pharmacological implications and must be taken into account when novel antagonist analogues are developed.

In contrast to vitamin D, ZK159222 was unable to cooperate with 9-*cis*-RA to stimulate either promoter activity (Fig 3A) or endogenous *cyp24* mRNA levels (Fig 3B) in cells expressing the AF-2 VDR mutant. This is also in accordance with the lack of coactivators recruitment observed in EMSAs. These assays showed the ability of this compound to recruit coactivators to the native heterodimer in the presence of 9-*cis*-RA, and this can be observed again in Fig 3C with the native TIF-2. However, vitamin D, but not ZK159222, cooperated with the RXR ligand when the second LXXLL motif of the coactivator was mutated. These results show that 9-*cis*-RA can overcome the effect of an AF-2 VDR mutation, compensate the effect of a deleterious mutation in the coactivator or even allow transcriptional activity by ZK159222, but the system does not tolerate more than one of these changes probably because stable coactivator binding to the receptors cannot be achieved under these conditions.

RXR ligands and differentiation of colon carcinoma cells

High concentrations of vitamin D promote differentiation of SW480-ADH colon carcinoma cells, and induction of E-cadherin gene transcription seems to have a key role in this response (Palmer *et al*, 2001). As shown in Fig 4A, 9-*cis*-RA (1 μM) or a low dose of vitamin D (3 nM) alone were unable to increase E-cadherin levels. However, a strong synergistic effect was found when both compounds were combined. This effect was also observed with the combination of the RXR ligand and ZK159222, showing again that this analogue presents a strong agonistic effect in the presence of 9-*cis*-RA. The combination of vitamin D with a low concentration of 9-*cis*-RA (10 nM) also synergistically increased E-cadherin expression (supplementary Fig 5 online).

Differentiation of SW480-ADH cells is characterized by the acquisition of an epithelial-like phenotype with formation of

compact islands with high E-cadherin expression at the cell membrane. In agreement with the E-cadherin levels, this process was not induced by any of the ligands individually, but the combination of the VDR ligands with the RXR ligand clearly induced differentiation (Fig 4B). Therefore, the role of the RXR ligand is also manifested in a complex response such as differentiation, showing its physiological importance in VDR-dependent responses.

METHODS

Plasmids. The multimerized DR3 (4xVDRE) AGGTCAtgaAGGACA is cloned in pGL3. Other reporters are described in supplementary Fig 1 online. Human RXRα and VDR are cloned in pSG5. Point mutations were obtained by PCR with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA). Glutathione *S*-transferase (GST)–activator of retinoic acid receptor (ACTR), GST–TIF-2, GST–steroid receptor coactivator 1 (SRC-1) and GST–DRIP205 code for the nuclear receptor-interacting domains of these proteins. These plasmids, as well as the His-tagged nuclear receptor-interacting domain of TIF-2 and mutants in the LXXLL motif I (M1), motif II (M2) and motif III (M3), have been described previously (Castillo *et al*, 2004).

Transfections. 293-T cells, grown in 24-well plates, were transfected with 40 ng of reporter using calcium phosphate, plated in medium containing hormone-stripped serum and treated after an overnight incubation. When appropriate, the reporter was co-transfected with RXR (12.5 ng) and wild-type or mutant VDR (12.5 ng) or empty vectors. Luciferase activity was determined after 36 h of treatment with 3 nM vitamin D, 3 nM ZK159222 and/or 1 μM 9-*cis*-RA, TTNPB or LG101208. Experiments were performed with triplicate cultures and each experiment was repeated at least three times. Data are represented as mean ± s.d. For siRNA experiments, 33 nM of a control or VDR siRNAs was transfected with Lipofectamine 2000. Transfected cells were incubated for 48 h before treatment. The efficiency of knockdown was determined by western blot (supplementary Fig 2 online).

RNA extraction. Total RNA was extracted using Tri Reagent (Sigma-Aldrich Química SA, Madrid, Spain). *cyp24* mRNA levels were analysed by quantitative reverse transcriptase–PCR using the primers 5′-GGCAACAGTTCTGGGTGAAT-3′ and 5′-TATTTGCG GACAATCCAACA-3′. Values were corrected by glyceraldehyde-3-phosphate dehydrogenase expression determined with the primers 5′-ACAGTCCATGCCATCACTGCC-3′ and 5′-CTAGCTGA CCTCCTTGACCTG-3′.

Gel retardation assays. EMSAs were performed as described previously (Castillo *et al*, 2004), with the single DR3 5′-AGGTC AAGGAGGTCA-3′ and 2.5 μl of *in vitro*-translated receptors (TNT Quick, Promega, Madison, WI, USA), in the presence and absence of 400–600 ng of GST-fused coactivators or 450 ng of His-tagged TIF-2. Vitamin D (1 μM), ZK159222 (1 μM) or 9-*cis*-RA (10 μM) was present in the assays as indicated. Sequences of other VDREs are shown in supplementary Fig 2 online.

Western blotting and immunostaining. SW480-ADH cells were grown as described previously (Palmer *et al*, 2001) and treated with vitamin D (3 nM), ZK159222 (30 nM) or 9-*cis*-RA (1 μM or 10 nM) for 48 h. Proteins (20 μg) were transferred to Immobilon P and probed sequentially with E-cadherin (BD Transduction Laboratories, BD Biosciences, San José, CA, USA, 1:1,000) and Lamin B1 (Santa Cruz Biotechnology, Heidelberg, Germany, 1:2,000) antibodies. For immunostaining, cells were incubated with a 1:100 dilution of the primary and secondary antibodies as described previously (Palmer *et al*, 2004).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

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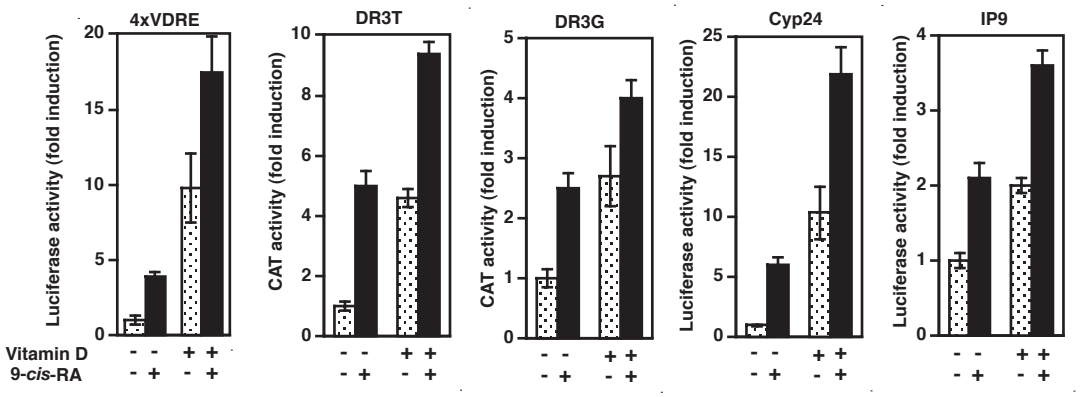
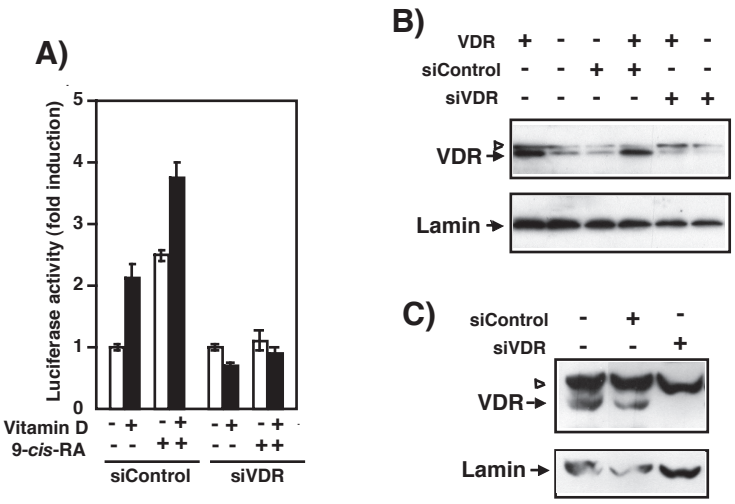
Supplementary Figure 1

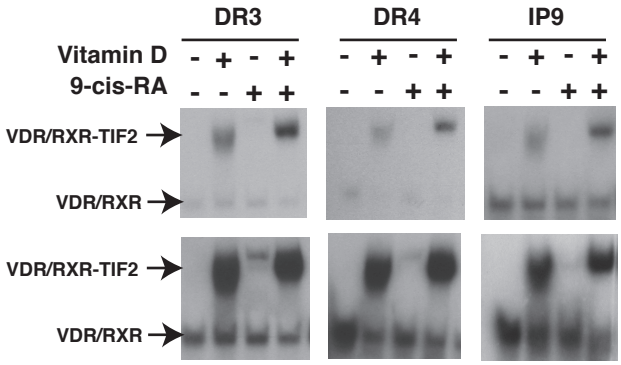


Cooperation of vitamin D and 9-*cis*-RA on different VDREs. The influence of the VDR and RXR ligands was analyzed in 293-T cells transiently transfected with VDR/RXR and various VDRE-containing reporter constructs. 4xVDRE is the luciferase reporter plasmid containing 4 copies of the DR3 (AGGTCAtgaAGGACA) present in the rat atrial natriuretic factor (ANF) promoter used in Fig.1. DR3T harbors the single response element GGTTCAcgaAGTTCA upstream of the thymidine kinase promoter driving expression of the chloramphenicol acetyl-transferase (CAT) gene. DR3G is similar, but contains the element AGGTCAaggAGGTCA. In the Cyp24 reporter, the -367 to +1 fragment of the human *cyp*24 gene is fused to luciferase. This promoter region contains two well-characterized VDREs in positions -293/-273 and -172/-143. IP9 is a luciferase reporter plasmid that harbors the sequence TGACCCtggaaaccgGGTCCA, a single palindromic element spaced by 9 oligonucleotides present in the mouse c-*fos* promoter. Regulation was similar in all cases: incubation with 9-*cis*-RA alone increased reporter activity and transactivation by vitamin D was further induced in the presence of the RXR ligand.

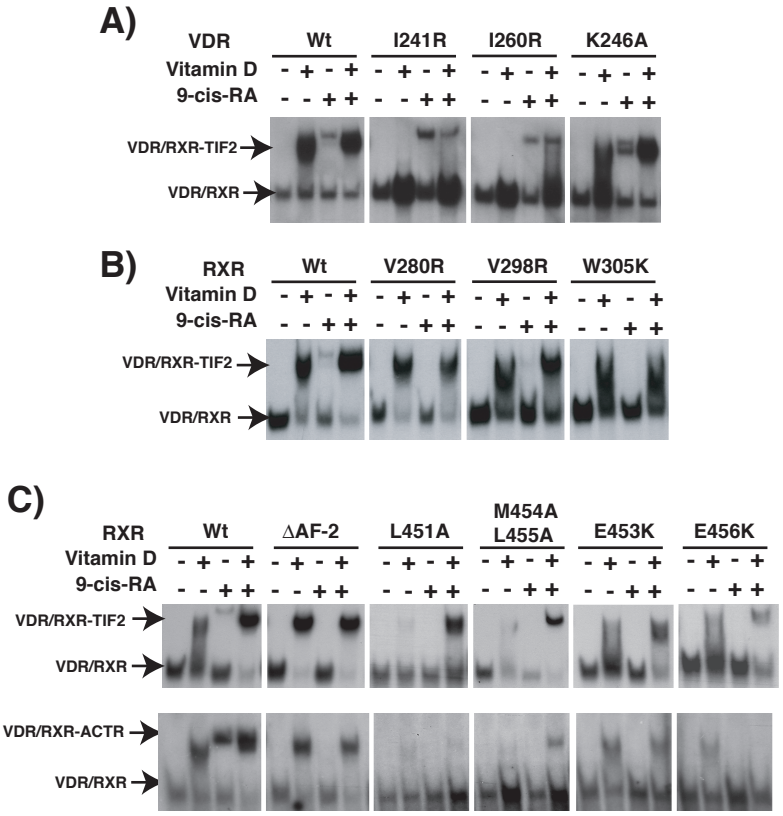


siRNA knock-down of VDR. In **panel A)** luciferase activity was determined in 293-T cells transfected with the *cyp*24 reporter plasmid described in Supplementary Fig.1 and the siRNA control (Dharmacon siCONTROL non-targeting siRNA #1) or the siRNA against VDR (Dharmacon siSMRT pool M-0034-48-00-005). Transfected cells were incubated for 48 h before treatment with vitamin D and or 9-*cis*-RA. Knock-down of endogenous VDR abolished *cyp*24 promoter stimulation by both ligands. In **Panel B**) VDR levels were analyzed by western blot in 293-T cells after 48 h of transfection with VDR or an empty vector and the siRNAs, as indicated. The empty arrowhead shows the position of a non-specific band. The VDR antibody (Chemicon) was used at a 1:1000 dilution. In the lower panel the blot was reprobed with an antibody against Lamin used as a loading control. **Panel C)** shows endogenous VDR and Lamin levels in MCF-7 cells transfected for 48 h with the control siRNA and the VDR siRNA.

Supplementary Fig.3

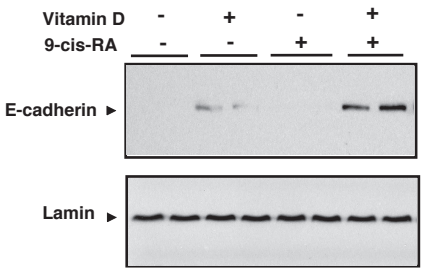


Gel retardation assays with various VDREs. In vitro translated VDR and RXR and the p160 coactivator TIF-2 fused to GST were used for band-shift assays with oligonucleotides conforming the consensus DR3-type VDRE agctcAGGTCAaggAGGTCAg, the DR4-type VDRE agcttAGTTCAtgagAGTTCAg identified in the rat Pit-1 gene, and the IP9 VDRE agctTTGCCTgggtgaatgAGGACAg of the rat osteocalcin promoter. Vitamin D and 9-*cis*-RA were present in the assays as indicated. The upper panels show low film exposures to illustrate cooperation of both ligands for coactivator recruitment. The lower panels show higher exposures suitable for observing recruitment by 9-*cis*-RA.



Recruitment of coactivators by point mutants of RXR and VDR in the coactivators binding surface. Gel retardation assays were performed with the DR3 oligonucleotide agctcAGGTCAaggAGGTCAg and p160 coactivators as indicated. In **panel A)** recruitment of TIF-2 in response to vitamin D and or 9-*cis*-RA was analyzed with wild type RXR, wild type VDR (Wt) and the VDR mutants I241R (helix 3), I260R (helix 4) and K246A (helix 3). The mutations I241R and I260R had a stronger effect than the K246A mutation and totally abolished recruitment by vitamin D and the synergism with 9-*cis*-RA, although recruitment by the retinoid was not affected. In **panel B)** was analyzed the effect of equivalent RXR point mutations V280R (helix 3) and V289R (helix 4), as well as mutation W305K (helix 5) on binding of TIF-2 to VDR/RXR. These mutations inhibited coactivator recruitment by 9-*cis*-RA and and in the case of the helix 4 and helix 5 mutants also reduced the response to vitamin D. **Panel C** shows the effects of mutations in helix 12 of RXR. RXR lacking helix 12 (ΔAF-2), the point mutants L451A, E453K and E456K, and the double mutant M454A/L455A were used to study association with ACTR (upper panel) and TIF-2 (lower panel). All mutations abolished recruitment of coactivators by 9-*cis*-RA, although synergism with vitamin D was observed in some cases. Additionally, whereas deletion of RXR helix 12 did not reduce recruitment of coactivators by vitamin D, association of ACTR or TIF-2 with the heterodimer in response to the VDR ligand was markedly reduced in the case of the point mutants. These results confirm the important role allosteric communication between VDR and RXR on binding of coactivators to the VDR/RXR heterodimer.

Supplementary Fig.5



Vitamin D cooperates with a low concentration of 9-*cis*-RA to increase expression of E-cadherin in colon cancer cells. E-cadherin levels were determined by western blot in SW480-ADH cells treated with 3 nM vitamin D in the presence and absence of 10 nM 9-*cis*-RA during 48 h (upper panel). The membrane was reprobed with anti-lamin antibody (lower panel).

VITAMIN D-DEPENDENT RECRUITMENT OF THE COREPRESSORS SMRT AND NCoR TO VITAMIN D RECEPTOR/RETINOID X RECEPTOR HETERODIMERS

Ruth Sánchez Martínez, Alberto Zambrano, Ana I. Castillo y Ana Aranda

Artículo enviado para su publicación

Introducción al Capítulo 3

Como parte final del estudio de la función del RXR y su ligando en la regulación transcripcional por TR y VDR, analizamos el papel del reclutamiento de correpresores en la señalización mediada por el heterodímero VDR/RXR. A diferencia de otros receptores no esteroideos, como TR y RAR, ni la capacidad represora de la transcripción, ni el reclutamiento de correpresores por VDR en ausencia de ligando, han sido claramente demostrados. Tampoco se ha descrito unión de correpresores en respuesta a antagonistas, como en el caso de los receptores esteroideos. En este trabajo mostramos por primera vez cómo el heterodímero VDR/RXR es capaz de reclutar los correpresores SMRT y NCoR en respuesta a un ligando agonista del VDR cuando se encuentra sobre un elemento de respuesta adecuado. Este hecho se ha comprobado tanto en ensayos de retardo en gel como en ensayos ChIP, que muestran la unión cíclica de estos correpresores y de HDAC3 al promotor del gen diana de la VitD, *cyp*24. Esta asociación del heterodímero con correpresores es claramente dependiente de agonista ya que se obtiene una fuerte correlación entre la unión de correpresores y el potencial agonístico de diversos análogos de la VitD tanto en ensayos de retardo en gel como en experimentos de "un híbrido" realizados en células 293-T. Para que se produzca el reclutamiento de correpresores al heterodímero en respuesta a agonistas de VDR además es indispensable la integridad de la H12 del VDR, en la que reside la activación transcripcional dependiente de ligando. La unión de un ligando al RXR, sea agonista o antagonista, produce sin embargo liberación de los correpresores unidos al heterodímero. Así ambos ligandos del heterodímero parecen tener papeles antagónicos en cuanto al reclutamiento de correpresores. Este reclutamiento en respuesta a agonistas de VDR se produce a través del RXR, ya que las mutaciones en los residuos que forman la superficie de interacción con correpresores del RXR muestran efectos drásticos sobre el reclutamiento, lo que no ocurre con las mutaciones equivalentes en el VDR. El reclutamiento de SMRT y NCoR al heterodímero se incrementa fuertemente tras la deleción de la H12 del RXR. Este aumento en la interacción se observa no sólo *in vitro*, sino en experimentos de "un híbrido" en células 293T. El heterodímero de VDR con el RXR carente de H12 (RXRΔH12) presenta una clara disminución en la activación transcripcional mediada por VitD, debido al incremento de la unión de correpresores. Esto se observa tanto en ensayos de transfección transitoria con construcciones reporteras que contienen VDREs, como analizando los niveles de RNA mensajero del gen *cyp*24. Puesto que la activación transcripcional por VitD está mediada por el reclutamiento de coactivadores analizamos además la implicación de los correpresores en la modulación de la respuesta transcripcional a la VitD. Los ensayos de competición nos indican que la unión de correpresores se desplaza tras la adición de coactivadores, demostrando la preferencia del heterodímero por la unión de estos últimos. Así nos planteamos si las respuestas transcripcionales a la VitD estarían determinadas por el balance celular entre ambos tipos de correguladores. Esto se comprobó mediante la inhibición de la expresión de los correpresores SMRT y NCoR por técnicas de siRNA. La disminución de los niveles de correpresores produce un aumento en la respuesta transcripcional a VitD tanto en ensayos de transfección transitoria como analizando los niveles de mensajero de *cyp*24. Además en ensayos ChIP, la depleción de los

correpresores produce un aumento de la acetilación de la histona H4 y una disminución del reclutamiento de HDAC3 en respuesta a VitD, reflejo del aumento de estimulación transcripcional.

En resumen, nuestros resultados muestran un nuevo modelo de reclutamiento de correpresores por los receptores nucleares, que se produciría en respuesta a la unión de un agonista. Ya que los coactivadores y correpresores compiten por la unión al heterodímero, el contenido relativo entre ambos tipos de correguladores podría modular de forma específica las respuestas transcripcionales a la VitD en los diferentes tejidos diana.

La alumna ha contribuido a este trabajo con la realización de ensayos EMSA, transfecciones, técnicas de siRNA y Western Blot. También ha realizado la mutagénesis de los receptores VDR y RXR y la caracterización funcional de los mismos. Ha contribuido a la puesta a punto de los ensayos de inmunoprecipitación de cromatina y al diseño de oligonucleótidos específicos para los mismos. Además de la parte técnica, la alumna ha intervenido en el planteamiento experimental y posterior discusión de los resultados mostrados en este artículo.

Vitamin D-dependent recruitment of the corepressors SMRT and NCoR to vitamin D receptor/retinoid X receptor heterodimers

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Running Title: Recruitment of corepressors by vitamin D

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Summary

Transcriptional regulation by nuclear receptors is mediated by recruitment of coactivators and corepressors. In the classical model, unliganded non-steroidal receptors bind corepressors, such as silencing mediator of thyroid and retinoid receptors (SMRT) or nuclear corepressor (NCoR) that are released upon ligand binding. We show here that, unlike other receptors, the heterodimer of the vitamin D receptor (VDR) with the retinoid X receptor (RXR) recruits NCoR and SMRT in a VDR agonist-dependent manner. Binding of an agonist to VDR allows its partner receptor, RXR, to bind the corepressors. 1,25-dihydroxy-vitamin D3 (vitamin D) causes in vivo recruitment of SMRT and NCoR to a VDR target promoter, and down-regulation of corepressors enhances transcriptional responses to vitamin D. These data reveal a new paradigm of SMRT and NCoR binding to nuclear receptors and demonstrate that these corepressors can function as physiological negative regulators of vitamin D-mediated transcription.

Introduction

Regulation of transcription by nuclear hormone receptors is mediated by recruitment of coregulators (coactivators and corepressors). Different models of corepressors recruitment have been identified. In the first one, unliganded receptors such as the thyroid hormone receptor (TR) or the retinoic acid receptor (RAR) act as strong constitutive repressors when bound to hormone response elements (HREs) in target genes, due to the binding of corepressors such as NCoR (nuclear receptor corepressor) or SMRT (silencing mediator of retinoic and thyroid receptor) (Chen and Evans, 1995; Horlein et al., 1995). NCoR and SMRT are large modular proteins that serve as platforms for formation of multicomponent repressor complexes that contain histone deacetylases (HDACs) and cause chromatin compactation (Guenther et al., 2000; Heinzel et al., 1997; Huang et al., 2000; Li et al., 2000; Nagy et al., 1997; Wen et al., 2000; Yoon et al., 2003). Ligand binding allows the release of corepressors and enables these receptors to recruit coactivators that cause chromatin decompactation and transcriptional stimulation.

Steroid hormone receptors would be the prototype for a second model. Unbound steroid hormone receptors do not interact effectively with the corepressors, and therefore do not have silencing activity. Binding of an agonist causes coactivator recruitment and activation. However, clear interactions both in vivo and in vitro were observed with receptor-bound antagonists (Smith and O'Malley, 2004). Therefore, antagonists can convert steroid receptors into transcriptional silencers. Structural studies have shown a shift in the position of the C-terminal helix (H12) of the ligand binding domain in the antagonist-bound estrogen receptor (ER) that not only disrupts coactivator interaction, but might also expose the surface for corepressor binding. This helix that contains the core ligand-dependent transcriptional activating domain (AF-2), sterically prevents corepressor binding to many nuclear receptors. For instance, the retinoid X receptor (RXR) has at most a weak silencing activity, but deletion of this region allows strong corepressor interaction and transforms RXR into a potent repressor (Zhang et al., 1999).

Many nuclear receptors require heterodimerization with RXR for high-affinity DNA binding and this receptor appears to play an active role in corepressor interaction (Zhang et al., 1997). It has been proposed that sequence-specific interactions between H12 of RXR and an hydrophobic cleft of unliganded TR formed by residues in H3, 4 and 5 repositions H12 and unmasks the corepressor interaction surface of RXR, allowing the unoccupied TR/RXR heterodimer to repress transcription. It is likely that RAR behaves similarly, but other RXR-heterodimerizing receptors would not present the interaction with RXR H12, thereby providing an explanation of variations in their repression function (Zhang et al., 1999).

In this work we show that binding of an agonist to the vitamin D receptor (VDR) induces association of the corepressors SMRT and NCoR to DNA bound VDR/RXR heterodimers, demonstrating the existence of a novel type of corepressor recruitment. ChIP assays show that corepressors are recruited in a ligand-dependent manner to the 1,25-dihydroxy-vitamin D3 (vitamin D) target gene *cyp*24, and knockdown of corepressors by means of siRNA enhances vitamin D-dependent transcriptional activity. Deletion of RXR H12 strongly enhances the ligand-dependent corepressor association and the heterodimer of VDR with the truncated RXR shows a markedly reduced vitamin D-dependent transcriptional response.

Opposite to that found with other heterodimers, enhanced binding of the corepressor requires an agonist-bound VDR and an intact VDR H12. Furthermore, although occupancy of VDR is responsible for corepressor recruitment, mutation of residues in H3, 4 and 5 of RXR (and not VDR) abolishes vitamin D-dependent corepressor recruitment and restores the transcriptional response to vitamin D. These results show that ligand binding to VDR triggers an allosteric change in the partner receptor that exposes its corepressor binding surface. Since VDR/RXR can recruit both coactivators and corepressors in an agonist-dependent manner, transcriptional activation by vitamin D may be modulated specifically in different target cells by the relative levels of these coregulators.

Results

Vitamin D-dependent association of corepressors to DNA-bound VDR/RXR heterodimers

In contrast with other receptors such as TR or RAR, unliganded VDR has only a weak silencing activity in 293-T cells transfected with an UAS reporter plasmid and GAL-VDR. Furthermore, over-expression of VDR/RXR neither represses the basal activity of reporter plasmids containing different VDRE motifs, nor decreases transcript levels of a vitamin D responsive gene such as *cyp*24 (supplementary Fig.1). This suggests that this receptor does not efficiently bind corepressors in the absence of ligand. To analyze this point, we first performed GST pull-down assays. As illustrated in Fig.1A, VDR showed detectable binding to the corepressor SMRT in this assays, but incubation with vitamin D did not cause the release of the corepressor. In contrast, triiodothyronine (T3) was able to dissociate SMRT from TR in the same assay.

To directly analyze in vivo binding of SMRT to a vitamin D target gene, ChIP assays were carried out in 293-T cells after expression of the VDR/RXR heterodimer. As shown in Fig.1B, SMRT binding to the *cyp*24 promoter was very weak but, surprisingly, the corepressor was recruited to the promoter in a vitamin D-dependent manner. Furthermore, HDAC3, a key component of the nuclear corepressor complexes (Guenther et al., 2000; Li et al., 2000; Wen et al., 2000; Yoon et al., 2003), was also recruited to the *cyp*24 promoter in response to vitamin D. On the other hand, and in agreement with the transcriptional stimulation secondary to coactivators recruitment, a net increase in the amount of acetylated H4 histone bound to the promoter was also found.

Corepressor function appears to be restricted by steric effects related to DNA binding. Thus, corepressors are able to interact strongly with the peroxisome proliferators activated receptors g (PPAR g) in solution but not on a PPRE, a context that reflects their in vivo function more accurately (Zamir et al., 1997). Therefore, we next examined recruitment of SMRT to the VDR/RXR heterodimer in gel retardation assays. Although in solution VDR is capable to bind SMRT, corepressor binding to the VDR/RXR heterodimer on a DR-3 VDRE was not observed (Fig.1C), which is more consisting with the finding that unliganded VDR does not repress transcription on this site. Additionally, in agreement with the in vivo recruitment of SMRT to the *cyp*24 promoter, a weak but detectable supershifted band was found upon incubation with vitamin D. This is not the case with the RXR ligand, *9-cis*-RA, that did not cause

corepressor recruitment. Since deletion of H12 in RXR allows strong corepressor interaction with this receptor and increases corepressor binding to PPARγ/RXR (Zamir et al., 1997; Zhang et al., 1999), we also analyzed binding of SMRT to the heterodimer of VDR with the H12-truncated RXR (RXRΔH12). Discernible SMRT binding to the mutant heterodimer was obtained in the absence of ligand (lane 7), and this binding was markedly enhanced in the presence of vitamin D (lane 9). Incubation with *9-cis*-RA had an opposite effect, causing SMRT release (lane 8). In contrast with steroid receptors in which antagonist but not agonist ligands induce corepressor recruitment, ZK159222, a VDR ligand with very low agonistic activity, possessed a strongly reduced ability to recruit SMRT when compared with vitamin D, suggesting an agonist-dependent binding of the corepressor to VDR/RXR. To further prove this point, we conducted gel retardation assays with a battery of vitamin D analogs with different agonistic activity (Castillo et al., 2006). The results obtained indeed demonstrated that only the full VDR agonists ZK161422 and ZK157202 caused a strong increase of SMRT association (Fig.1D). However, as shown in Fig.1E, both the rexinoid LG100268 and the RXR-selective antagonist LG101208 were able to cause corepressor release from the vitamin D-bound heterodimer, showing that an agonist conformation of RXR is not required to revert SMRT binding. The VDR agonist-dependent recruitment of corepressors was not restricted to SMRT, since the heterodimer also bound NCoR in a vitamin D-dependent manner (Fig.1F).

It has been shown that the length of H12, rather than its primary sequence or amphipathicity is the critical factor in RXR's inability to interact with corepressors and repress transcription (Zhang et al., 1999). This also appears to be true for the increased corepressor recruitment by VDR/RXR, since the strong SMRT recruitment by the H12-deleted heterodimer was not observed with different point mutations in this helix (Fig.1G).

The carboxyl terminus of SMRT possesses two receptor interacting domains (ID1 and ID2). These domains, also named CoRNR boxes, each contain the motif I/LXXI/VI (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999). Since the IDs differ in their affinity for specific receptors (Ghosh et al., 2002; Wong and Privalsky, 1998), we also analyzed the effect of individual and combined mutations in these domains. As shown in Fig 1G, mutation of any of the IDs abolished vitamin D-dependent SMRT recruitment to the heterodimer.

Although a DR3 is a common VDRE, VDR/RXR can also bind to elements with other configurations. SMRT binding in response to vitamin D was also found using DR4 and IP-9 response elements (supplementary Fig.2).

Role of VDR H12 on SMRT recruitment

Since recruitment of SMRT to the DNA-bound heterodimers appears to be dependent on the binding of an agonist to VDR, we next analyzed the effect of mutations in the AF-2 domain of this receptor on association with the corepressor. The conformational change elicited in the receptors by agonist binding involves repositioning of H12. This helix folds back against the ligand binding pocket and together with residues is H3, 4, and 5 generates a hydrophobic cleft responsible for coactivator interaction. A conserved glutamic acid residue in H12 (E420 in VDR) and an invariable lysine residue in H3 (K246 in

VDR) contact directly with the coactivator and form a charge clamp that stabilizes binding (for a review see Aranda and Pascual; 2001). We have previously shown that deletion of H12 (VDRΔH12) or mutations E420Q and K246A abolish vitamin D-dependent binding of coactivators and transcriptional activity (Jimenez-Lara and Aranda, 1999; Sanchez-Martinez et al., 2006). As shown in Fig.2A, the H12 mutants also exhibited a distinct reduction of SMRT association in response to vitamin D. In contrast, the K246A mutant showed an increased corepressor binding that was noticeable in the presence of *9-cis*-RA. Therefore, H12 of VDR also has an important role on SMRT recruitment, but the charge clamp involved for coactivator binding appears to be dispensable for corepressor binding.

This pattern of SMRT recruitment was very different from that obtained with the heterodimer of RXR with TR. Deletion of RXR H12 also caused a remarkable enhancement of SMRT binding by TR/RXR, but this increase was ligand-independent and occupancy of TR by T3 caused some release of SMRT that was potentiated by *9-cis*-RA. Furthermore, mutation of TR H12 in the glutamic acid residue equivalent to E420 in VDR did not reduce corepressor binding, but rather inhibited significantly SMRT dissociation by both ligands (Fig.2B).

In vivo binding of SMRT to VDR/RXR and VDR/RXRΔH12

In vivo binding of SMRT to the wild type and mutant VDR/RXR heterodimer was analyzed by "one-hybrid" assays in 293-T cells (Figure 3A). In these assays transactivation of the VDRE reporter by vitamin D was examined in cells transfected with the receptors and the VP16 activation domain alone or fused to the C-terminus of SMRT that contains both IDs (VP16-SMRT). Whereas expression of VP16 alone did not increase transactivation by vitamin D, this response was enhanced significantly by VP16-SMRT, confirming the ligand-dependent interaction of the corepressor with the receptors observed in the in vitro assays. Also in agreement with the results obtained in the supershift assays, deletion of RXR H12 caused a strong increase of in vivo binding of SMRT to the heterodimer. Similar results were observed with NCoR (supplementary Fig.3). Of interest, the increased association with corepressors caused a marked reduction of the response to vitamin D mediated by VDR/RXRΔH12.

To analyze whether in vivo association with SMRT was also agonist-dependent, the ability of VP16-SMRT to increase transactivation by VDR ligands with different activity was tested. As shown in Fig.3B, there was a very good correlation between the agonistic activity of various vitamin D analogs and the interaction with SMRT in "one hybrid" assays. As corresponding with the stronger binding of the corepressor to VDR/RXRΔH12, the slope of the regression line was higher with the mutant that the wild type receptors that, however, mediated a stronger transactivation by the VDR ligands.

In contrast with the results obtained in the "one hybrid" assays performed with the receptor heterodimer and the VDRE, GAL-SMRT did not affect vitamin D-dependent transactivation of a UAS-containing plasmid by VP16-VDR. This result agrees with the lack of vitamin D-dependent effect on SMRT binding obtained in pull-down assays, and suggests an important role of RXR in this interaction. Furthermore, when similar assays were performed in the presence of RXR or RXRΔH12, vitamin D was still unable to induce association with VP16-SMRT (supplementary Fig.4). This demonstrates that not only

both partner receptors are required for corepressor recruitment, but that their DNA binding domains need to be attached to an appropriate DNA response element to allow ligand-dependent SMRT recruitment.

Deletion of RXR H12 reduces vitamin D-mediated transcription

As described above, deletion of RXR H12 reduces the transcriptional response to vitamin D in transactivation assays. If this is related to the increased SMRT binding, incubation with *9-cis*-RA, that causes dissociation of the corepressor, should have a stimulatory effect. Fig.4A shows that the response to vitamin D mediated by the truncated heterodimer was reversed to a significant extent when the vitamin was combined with the RXR ligand. In contrast to that found with the native heterodimer, *9-cis*-RA does not cause coactivator recruitment to VDR/RXRΔH12 (Sanchez-Martinez et al., 2006). Therefore, the increase by this ligand should be secondary to corepressor release. Increased association with SMRT by deletion of RXR H12 also produced a clear reduction of the ability of vitamin D to induce transcription from an endogenous gene. As shown in Fig.4B, transcripts from the *cyp*24 gene were strongly induced by vitamin D in 293-T cells upon expression of VDR/RXR and this response was markedly blunted when VDR/RXRΔH12 was expressed. In addition, *9-cis*-RA cooperated with vitamin D to induce *cyp*24 mRNA levels and the reduced response from VDR/RXRΔH12 was significantly recovered.

Since incubation with vitamin D also causes the recruitment of coactivators to the heterodimer, we next examined the effect of the p160 coactivators TIF-2 and ACTR on SMRT binding. As can be observed in Fig.4C, the mobility of the ternary complex of the receptor heterodimer with p160 coactivators was slower than that obtained with SMRT, and the coactivators displaced binding of the corepressor. These results suggest that the coactivator/corepressor ratio should determine vitamin D-mediated transcriptional activity. If this is the case, high coactivator levels should revert the reduced activity of the truncated heterodimer that shows a strongly increased association with corepressors, whereas transactivation by vitamin D should be further reduced in cells expressing high corepressor levels. Accordingly, overexpression of TIF-2 was able to revert almost totally the reduced transcriptional activity of the heterodimer of VDR with RXR lacking H12, whereas transactivation by vitamin D was further reduced upon transfection with a SMRT expression vector (Fig.4D).

SMRT binds to RXR in the heterodimer

Association of corepressors to TR requires the conserved residues A, H and T in the so called CoR box located in H1 (Horlein et al., 1995), and mutation of the homologous amino acids (AEV) in RXR lacking H12 abrogates interaction with this receptor (Zhang et al., 1999). However, the AEV mutant was able to interact normally with SMRT in the context of the VDR heterodimer, showing that this region of RXR is dispensable for corepressor association in response to vitamin D (Fig.5A). However, CoR box residues are not exposed in the LBD surface and the corepressor does not interact directly with this TR region, but docks to a hydrophobic groove formed by H3, 4 and 5 that overlaps partially with the coactivator interacting domain (Hu and Lazar, 1999; Marimuthu et al., 2002; Nagy et al., 1999; Perissi et al., 1999). We then tested the effect of mutations in the putative corepressor binding surface of RXR on

vitamin D-dependent SMRT association. Mutations V280R in H3, V298R in H4 and W305K in H5 (equivalent to those that abrogate NCoR binding to TR), abolished recruitment of SMRT to the VDR/RXRΔH12 heterodimer in response to vitamin D (Fig.5A), showing that the corepressor binds to this moiety of the heterodimer.

To analyze the role of VDR on corepressor recruitment by the heterodimer, the effect of mutations in the CoR box (AHT) (Horlein et al., 1995) and in P122, a residue preceding H1 equivalent to P214 in TRβ required for corepressor binding (Pissios et al., 2000) was also tested. As shown in Fig.5B, association of SMRT was not reduced in the P122R mutant, and only a slight reduction was observed with the CoR mutant. Similarly, the effect of mutations I241R in H3 and I260R in H4 was much weaker than that observed with the corresponding amino acids in RXR that abrogated interaction with the corepressor. Therefore, in contrast with the prominent role of the corepressor binding surface of RXR, this surface of VDR is not required for SMRT recruitment by vitamin D.

Mutation of H3, 4 and 5 in RXRΔH12 blocks in vivo binding of SMRT and restores vitamin D-dependent transcriptional activity.

Since mutations in the corepressor binding surface of RXR blocked interaction of the heterodimer with SMRT in response to vitamin D, it was expected that these mutations could restore the transcriptional activity the heterodimer of VDR with RXR lacking H12. Indeed, as shown in Fig.5C, mutations V280R, V298R and W305K in the context of RXRΔH12 enhanced transactivation by vitamin D reaching the levels obtained with the native receptors. That these receptors failed to interact in vivo with the corepressor is demonstrated by the finding that VP16-SMRT was unable to enhance the vitamin D response. Confirming the importance of corepressor binding, the AEV mutant that still interacted normally with VP16-SMRT also presented a reduced transcriptional activity.

The ability of the H3, 4 and 5 mutants in the context of the H12-deleted heterodimer to mediate vitamin D and *9-cis*-RA induction of endogenous *cyp*24 transcripts was also examined. As illustrated in Fig.5D, these mutations restored significantly the response to vitamin D with respect to that obtained with RXRΔH12. In addition, these mutants did not mediate transcription by *9-cis*-RA and cooperation of both ligands was not observed. This contrasts with the results obtained with RXRΔH12, the wild type receptors and the AEV mutant in which the effect of vitamin D was further enhanced by *9-cis*-RA. This different behavior could be a consequence of corepressor release that cannot occur after mutation of the RXR corepressor binding surface.

Role of endogenous corepressors on vitamin D-dependent transcription

siRNA was used to directly address whether endogenous SMRT and NCoR can function as negative regulators of vitamin D transcriptional activity. With this purpose 293-T cells were cotransfected with VDR/RXR heterodimers and control, SMRT or NCoR siRNAs. Corepressor levels were reduced by the corresponding siRNAs (Fig. 6C), and as shown in Fig. 6A, transactivation by vitamin D was enhanced by the SMRT and NCoR siRNAs, with respect to that found with the cells transfected with the control siRNA.

As shown in Fig.6B, siRNAs for both corepressors also increased ligand-dependent transcription of the *cyp*24 gene, although in the case of the endogenous vitamin D-responsive gene down-regulation of NCoR had a much stronger effect.

The recruitment of corepressors to the *cyp*24 promoter, as well as the effect of the siRNAs was also tested in MCF-7 cells that express high endogenous receptor levels and are highly responsive to vitamin D. ChIP assays (Fig.7A), demonstrated that both SMRT and NCoR are recruited to the target gene in a cyclic and agonist-dependent manner. Furthermore, as already observed in 293-T cells, HDAC3 was recruited with similar kinetics and enrichment on acetylated H4 histone was also observed in MCF-7 cells. As shown in Fig.7B, in these cells the SMRT antibody recognizes several bands that are strongly reduced in the presence of the specific siRNA, and NCoR levels are also markedly down-regulated by its siRNA. Under these conditions, a strong increase in acetylated histone H4, as well as a concomitant reduction on HDAC3 recruitment to the promoter was found (Fig.7C), supporting the hypothesis that endogenous corepressors function to suppress transcription by vitamin D-liganded VDR/RXR heterodimers.

DISCUSSION

In the classical model unoccupied receptors bind SMRT and NCoR that are released upon ligand binding. More recent results have demonstrated binding of these coregulators to antagonist-bound receptors. Our work shows that the VDR/RXR heterodimer represents a third paradigm for SMRT and NCoR binding. This heterodimer does not bind corepressors in the absence of ligand, but can recruit SMRT and NCoR in response to vitamin D. Although agonist binding to VDR triggers corepressors recruitment, the corepressor binding surface of RXR is used by these proteins to associate with the heterodimer. Since coactivators and corepressors compete with each other for binding to VDR/RXR in response to vitamin D, the cellular ratio of coregulators can modulate specifically transcriptional responses to vitamin D in different target cells.

Recruitment of corepressors by VDR/RXR is dependent on binding of an agonist ligand to VDR

The ability of the unoccupied VDR to bind SMRT and NCoR and to silence transcription has been controversial (Herdick and Carlberg, 2000; Horlein et al., 1995; Tagami et al., 1998). Our results show that although VDR can interact with these corepressors in pull-down assays, the unoccupied VDR has at most a weak silencing activity. In fact, the ability of this receptor to interact with NCoR and SMRT in the absence of ligand appears to be suppressed by heterodimerization with RXR, and opposite to that observed with other receptors such as TR or RAR (Wong and Privalsky, 1998; Zamir et al., 1997), no detectable binding of the corepressors with the unoccupied heterodimer is observed in in vitro or in vivo assays. However, our data demonstrate that this heterodimer can recruit the corepressors upon VDR occupancy, existing a very strong correlation between agonistic activity and corepressor binding. This is also different for that found with other nuclear receptors such as steroid receptors or PPARs, in which antagonist but not agonist

ligands enhance corepressor binding.

Most important, SMRT and NCoR are recruited in vivo to a target gene in a vitamin D-dependent manner, as observed with the *cyp*24 promoter in ChIP assays. This occupancy is cyclical, as has been previously observed for ER and coregulators (Metivier et al., 2003) and also occurs with VDR (Vaisanen et al., 2005). Recruitment of HDAC3, that is found in a tight complex with SMRT and NCoR (Guenther et al., 2000; Li et al., 2000) follows similar kinetics, showing that the corepressor complexes are recruited to the target gene in response to vitamin D. In vivo binding of corepressors and the deacetylase to the VDRE-containing promoter is opposite to that obtained in genes containing binding sites for other receptor heterodimers such as TR/RXR or PPAR/RXR, where HDAC3 as well as SMRT and NCoR are recruited in the absence of ligand and released upon ligand binding (Guan et al., 2005; Li et al., 2002). This mirror image matches the different model of corepressor recruitment by VDR/RXR and other heterodimers.

Interestingly, it has been recently demonstrated (Fujiki et al., 2005; Murayama et al., 2004) that NCoR corepressor complex components are recruited to the 1α (OH)ase promoter after incubation with vitamin D. This is a key enzyme in vitamin D biosynthesis that possesses a negative VDRE to which the receptors are tethered through interaction with a bHLH activator (Murayama et al., 2004). At the light of our present results this recruitment would not be specific for the transrepressed gene, but could rather reflect the intrinsic property of the heterodimer to associate with corepressors in the presence of vitamin D.

Role of H12 of RXR and VDR on corepressors recruitment by VDR/RXR

H12 of RXR plays an inhibitory role on vitamin D-dependent corepressors recruitment by VDR/RXR, since deletion of this helix greatly enhances the ability of the heterodimer to bind SMRT and NCoR. The increased association with corepressors correlates with the strongly reduced transcriptional activity of vitamin D through this heterodimer. Unlike that found with other heterodimers in which deletion of RXR H12 increases corepressor recruitment in the absence of ligand that is reversed upon ligand binding (Schulman et al., 1996; Zhang et al., 1999), heterodimerization with VDR masks the ability of truncated RXR to bind SMRT, and strong binding of the corepressor only occurs upon VDR occupancy.

In contrast with the inhibitory role of RXR H12 for corepressor binding, the integrity of VDR H12 is required for efficient binding of corepressors to the heterodimer in response to vitamin D. In agreement with the fact that this binding correlates with the agonistic activity of VDR ligands, deletion of mutation of this helix that blocks vitamin D-dependent recruitment of coactivators (Jimenez-Lara and Aranda, 1999; Sanchez-Martinez et al., 2006), also inhibits corepressor binding. This demonstrates that an agonist conformation of VDR, that implies repositioning of H12 is required for association of the corepressors to the heterodimer. Not surprisingly, the structural requirements for binding of coactivators and corepressors appear to be different, as indicated by the dispensability of the charge clamp between H3 and H12 for corepressors association that is however crucial for coactivators recruitment. Again the role of H12 of VDR is different to that of TR or RAR on corepressors recruitment. In the case of the later receptors mutation or deletion of H12, rather than inhibiting corepressor binding, potentiates association of the heterodimers

with the corepressors and reduces the ligand ability to dismiss these proteins (Chen and Evans, 1995; Lin et al., 1997).

Corepressors associate with RXR in response to ligand binding to VDR

Since corepressors recruitment by VDR/RXR is obtained in response to vitamin D and was dependent on the integrity of the VDR H12, intuitively looks as if corepressors would bind to this receptor as a consequence of the repositioning of this helix to the agonist position. However, mutation of the putative corepressor binding surface of this receptor, formed by the hydrophobic groove created by helices 3, 4 and 5 (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999), has little if any effect on recruitment. In contrast, mutation of the same surface in RXR abolishes corepressors recruitment, demonstrating that they are bound to this receptor. This indicates the existence of an important allosteric communication between both members of the heterodimer by which the conformational change elicited by ligand binding to one receptor is transmitted to its partner, a mechanism reminiscent of the "phantom ligand" effect observed with LXR/RXR (Willy and Mangelsdorf, 1997), in which the activation domain of LXR is required for stimulation by the RXR ligand. Crystals for VDR/RXR are presently unavailable and the structural basis for this change is presently unknown. However, it is clear that H12 of both receptors play a role in this interactions and crystal structure of other receptor dimers and heterodimers has demonstrated docking of H12 of one receptor within its partner (Westin et al., 1998). Our results are compatible with a model in which the shift in position of VDR H12 upon ligand binding unmasks the corepressor binding surface of RXR. This domain in TR would have an inverse function, namely to reposition H12 in RXR to mask this surface and to release the corepressor (Zhang et al., 1999). Of interest, in contrast to that observed with RXR homodimers, mutation of the RXR CoR box does not inhibit vitamin D-dependent corepressor recruitment, showing that in the heterodimeric context this region is dispensable.

The RXR ligand causes corepressors dismissal

Unlike binding of vitamin D to VDR, binding of *9-cis*-RA to RXR reverses SMRT and NCoR association with VDR/RXR. Not only the natural ligand, but also an antagonist induces this event, indicating that occupancy of the RXR ligand binding pocket but not acquisition of an agonist configuration is required for corepressor release. Since the RXR ligand is able to induce corepressor release in the absence of H12, reorganization of other receptor domains appears to be sufficient for this phenomenon. The release of corepressors by the truncated heterodimer has a clear functional effect. *9-cis*-RA still has the ability to induce transcription and to cooperate with vitamin D, despite the fact that the VDR/RXRΔAF-2 heterodimer is unable to recruit coactivators in response to the RXR ligand (Sanchez-Martinez et al., 2006). These transcriptional actions of *9-cis*-RA are lost in mutant receptors in the corepressor binding surface, demonstrating that the stimulatory effect of this ligand is indeed due to corepressor release. That corepressors dismissal, besides coactivators recruitment, could also contribute to stimulation of transcription by the wild type heterodimer in response to *9-cis*-RA should not be excluded.

Functional role of NCoR and SMRT on vitamin D- dependent transcription

Ligand binding is associated with recruitment of coactivators and activation of target gene transcription. Therefore, that also SMRT and NCoR could be recruited to VDR/RXR upon vitamin D binding is perhaps counterintuitive. However, the existence of corepressors such as RIP140 or LCoR that recognize many agonist-bound receptors through LXXLL motifs and play an important role in nuclear receptor functions in vivo is well documented (Fernandes et al., 2003; Steel et al., 2005).

Mutation analysis of TR, later confirmed in a crystallographic study with PPARa (Marimuthu et al., 2002; Xu et al., 2002) demonstrates that corepressors dock into the hydrophobic groove that also belongs to the coactivators-binding site, predicting that binding of coactivators and corepressors is mutually exclusive. Therefore, corepressors could compete with coactivators and function in a negative feedback loop to attenuate vitamin D-induced transactivation. The role of endogenous SMRT and NCoR in VDR/RXR action was shown by siRNA experiments, with corepressor down-regulation causing an increase in vitamin D transcriptional activity. Therefore, the relative expression of SMRT and NCoR versus coactivators may regulate distinct vitamin D responses in different target tissues.

Agonist-dependent corepressors recruitment may have additional roles. It can also function in ligand-dependent target gene repression, and may act as part of a cycle of cofactors recruited to target promoters by ligand-bound receptors. Receptors and coregulators rapidly cycle on and off target promoters (Metivier et al., 2003). This cycling is accompanied by waves of histone acetylation and deacetylation. Recruitment of corepressors may contribute to promoter clearance and deacetylation.

Experimental Procedures

Plasmids

Expression vectors for human VDR, RXRα, VDRΔH12, K246A, E420Q, and RXRΔH12 were described earlier (Castillo et al., 2004; Jimenez-Lara and Aranda, 1999). Other mutants were obtained by PCR with PfuTurbo DNA polymerase (Stratagene). All mutations were confirmed by direct sequencing. The luciferase reporter 4xVDRE contains a multimerized DR3 (AGGTCAtgaAGGACA). In the *cyp*24 reporter plasmid, a gift from S. Kato, the -367 to +1 fragment of the human *cyp*24 promoter (that contains two VDREs at -293/273 and -172/143) is fused to luciferase. Gal4-VDR contains the yeast Gal4-DBD fused to the receptor LBD (118-427) and was a gift of R. M. Evans. The reporter UAS plasmid contains Gal4 DNA-binding sites upstream of the E1B-TATA element fused to luciferase. The VP16 activation domain alone or fused to the C-terminus of SMRT was obtained from A. Baniahmad. The constructs GST-SMRT, GST-NCoR and GST-ACTR code for the nuclear receptor-interacting domains of these proteins and have been described previously (Castillo *et al.*, 2004). The His-tagged nuclear receptor-interacting domain of TIF-2 has also been described (Germain et al., 2002). The GST-SMRT M1 (V2142A/I2143A), M2 (I2345A/I2346A), and M1/2 mutants were constructed by site directed mutagenesis.

Transfections

293-T cells, grown in 24 wells plates, were transfected with 40 ng of reporter and 10 ng of pRL-TK-Renilla (Promega) as a normalizer control using calcium phosphate. After transfection cells were plated in medium containing hormone-stripped serum and after an overnight incubation treatments were started. When appropriate, the reporter was cotransfected with expression vectors for RXR (12.5 ng), wild type or mutant VDR (12.5 ng), TIF-2 or SMRT (10 ng) or with an equivalent amount of empty vectors. Unless otherwise stated, luciferase activity was determined after 36 hours of treatment with 3 nM vitamin D or vitamin D analogs and/or 1 μM *9-cis*-RA. Experiments were performed with triplicate cultures and each experiment was repeated at least 3 times. Data are represented as means ± standard deviations. Short-interfering RNAs for NCoR, SMRT and Non-target control were purchased from Dharmacon (Cat. M-003518-01, M-020145-01 and D-001210-01). siRNA transfections were performed using 33 nM of each siRNA and lipofectamine™2000 (Invitrogen) as recommended by the manufacturer. The efficiency of knockdown was determined by Western Blot.

RNA extraction and quantification

Total RNA was extracted using Tri Reagent (Sigma) and *cyp*24 mRNA levels were analyzed by quantitative RT-PCR as previously described (Sanchez-Martínez *et al.*, 2006).

Gel retardation assays

Assays were performed as described (Castillo *et al.*, 2004), with the DR3 oligonucleotide 5´-AGCTCAGGTCAAGGAGGTCAG-3´ and 2.5 μl of in vitro translated receptors (TNT Quick, Promega), in the presence and absence of 1 μg of GST-fused corepressors and 150, 250 or 400 ng of GST-ACTR or His-tagged TIF-2. Vitamin D and vitamin D analogs (1 μM) or *9-cis*-RA (10 μM) were present in the assays as indicated.

GST-pull down

Pull down assays were performed with 5 μl of in vitro translated ^{35}S-methionine-labeled VDR or TR (TNT-T7, Quick Transcription/Translation System™, Promega) and 1 μg of GST-SMRT or GST alone as previously described (Jimenez-Lara and Aranda, 1999). When indicated, 1 μM vitamin D or T3 were included in the binding reaction.

ChIP assays

293-T transfected with VDR and RXR and MCF-7 cells growing in p200 dishes were washed twice in serum-free medium and treated for 2.5 hours with 2.5 mM α-amanitin. Cells were then washed and treated with 3 nM Vitamin D. At the indicated time points, cells were fixed with 1% formaldehyde for 15 min/37 ºC. The Chromatin Immunoprecipitation Assay kit from Upstate (Cat. 17-295) was used. Sonication was performed using a Bioruptor UCD-200TM (Diagenode) following manufacturer's directions. For each immunoprecipitation 2.5-3.0x10^6 cells and 2 μg of the following antibodies: anti-

acetylated Histone 4 (Cat. 06-598, Upstate), anti-HDAC3 (Ab2379, Abcam), anti-SMRT (Cat. PA1-842, Affinity BioReagents), anti-NCoR (sc-8994, Santa Cruz Biotechnology) and normal rabbit serum immunoglobulins (sc-2027, Santa Cruz Biotechnology) were used. DNAs were subjected to 35 cycles of PCR with primers 5´-TGCCTTCCTGGGGGTTATCTC-3´ and 5´-CGTTTCCTCCTGTCCCTCTC-3´ that amplify a 286 bp segment of the *cyp*24 promoter.

Western

Twenty μg of total protein were separated on 5% SDS-PAGE and probed with specific primary antibodies and horseradish-coupled anti-rabbit or anti-mouse IgG (Cat. sc-2030 and sc-2005, Santa Cruz Biotechnology) as secondary antibodies. The primary antibodies used were anti-SMRT (Cat. PA1-842, Affinity BioReagents), anti-NCoR (Cat. 06-892, Upstate) at 2μg/ml, and anti-βcatenin (1/2000) (Cat. 610184, BD Biosciences Phar.) as a loading control.

Acknowledgments

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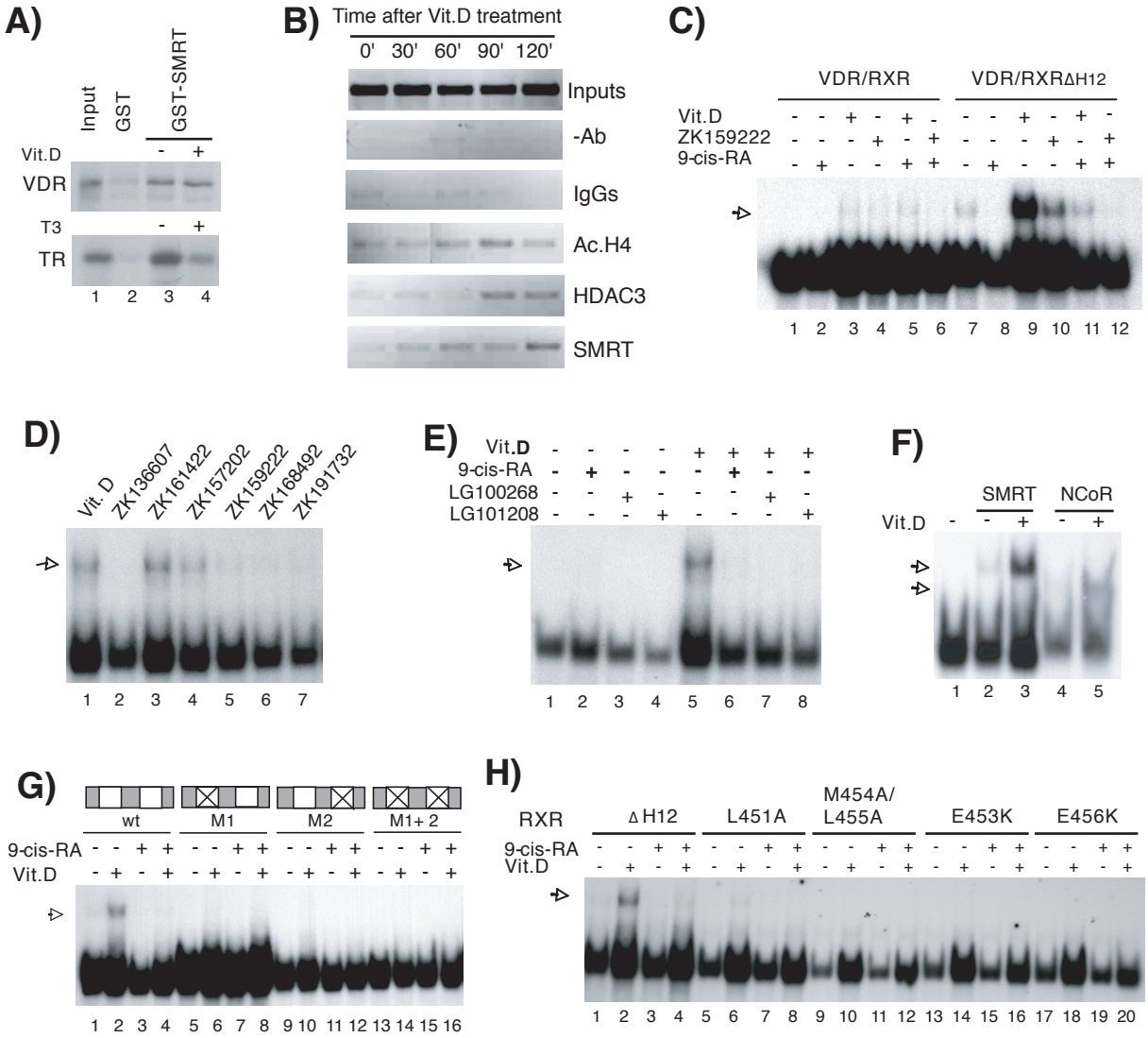

Figure 1. Vitamin D dependent recruitment of corepressors to VDR/RXR. (A) Pull-down assays with GST-SMRT and in vitro translated VDR or TR in the presence and absence of their corresponding ligands vitamin D and T3. (B) 293-T cells were transfected with VDR/RXR and 48 h later treated with vitamin D for the times indicated. ChIP assays were performed with antibodies against acetylated histone H4, HDAC3, SMRT or a control IgG. –Ab: minus antibody (C) Gel retardation assays with VDR/RXR or VDR/RXRΔH12 and SMRT in the presence of vitamin D, 9-*cis*-RA or the vitamin D analog ZK159222. (D) Gel retardation assays with VDR/RXRΔH12 in the presence of various VDR ligands with different agonistic activity. (E) Assays performed in the presence of vitamin D, 9-*cis*-RA, the RXR agonist LG100268 and the RXR antagonist LG101208. (F) Binding of SMRT and NCoR to VDR/RXR ΔH12 in the presence and absence of vitamin D. (G) Binding of wild type (wt) SMRT and mutants in the first (M1), second (M2) and both (M1+2) receptor interacting domains to VDR/RXRΔH12. (H) Comparison of SMRT binding to the heterodimer of VDR with RXR lacking H12 and different point mutants in this helix.The terciary complex containing the heterodimers and the corepresor is shown by an arrow.

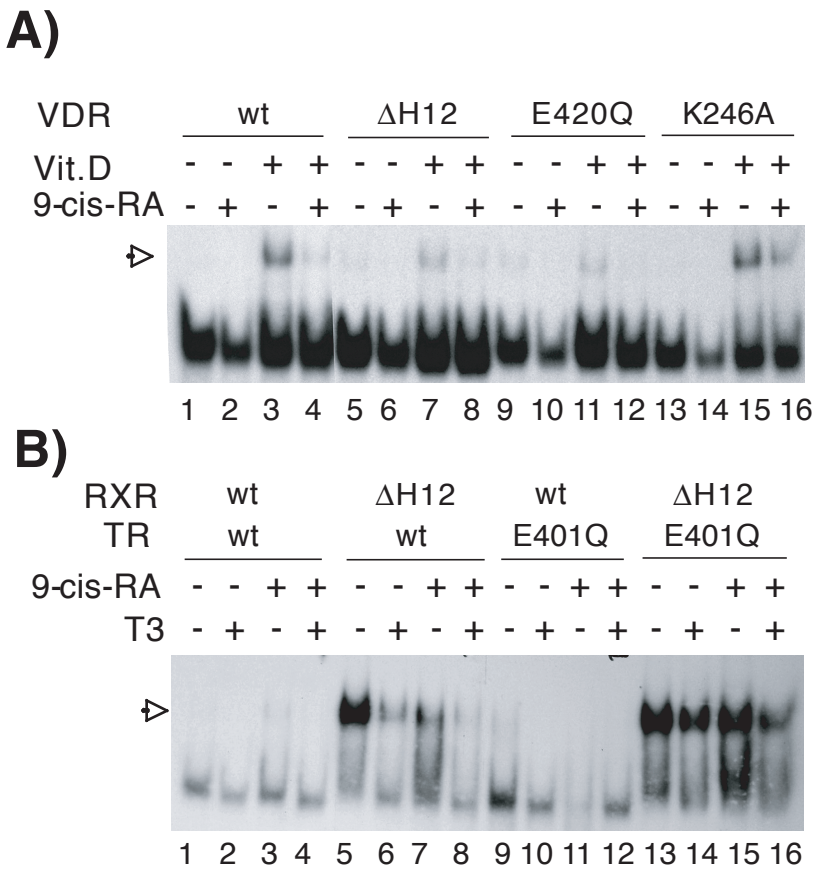

Figure 2. Role of VDR and TR AF-2 domains on SMRT binding. (A) Gel retardation assays with the heterodimers of RXRΔH12 with wild type VDR, VDRΔH12 and the point AF-2 mutants E420Q (in H12) and K246A (in H3). Assays were performed in the presence and absence of vitamin D and 9-*cis*-RA. (B) Binding of SMRT to the heterodimers of TR and the TRE401Q mutant with RXR and RXRΔH12. T3 and/or 9-*cis*-RA were included in the assays as indicated.

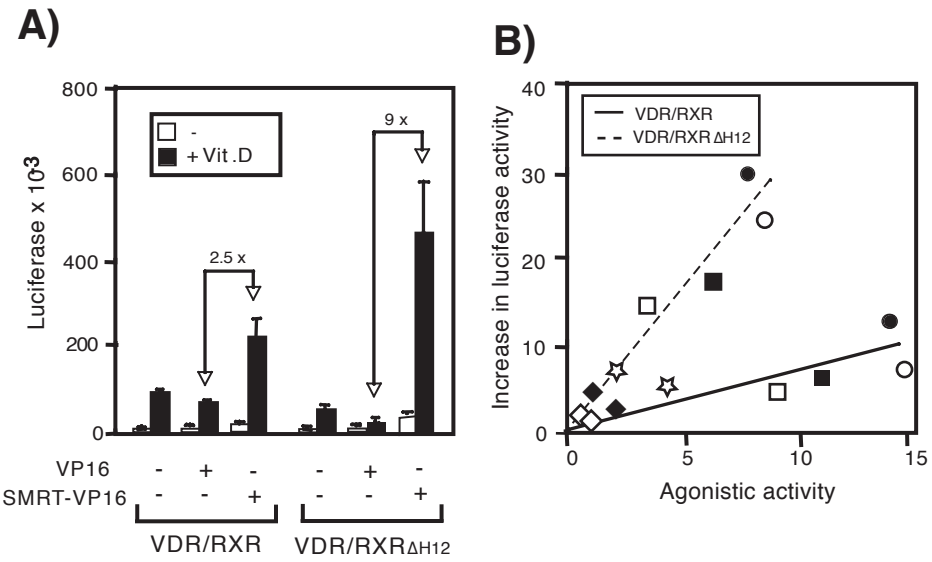

Figure 3. Vitamin D causes in vivo binding of SMRT to VDR/RXR. (A) One hybrid assays in 293-T cells cotransfected with the 4xVDRE-reporter plasmid, VDR/RXR or VDR/RXRΔH12 and the activation domain of VP16 alone or fused to SMRT. Luciferase activity was determined in cells incubated with and without vitamin D for 36 h. (B) The effect of vitamin D and various VDR ligands with different agonistic activity on binding of both heterodimers to SMRT was tested in one hybrid assays. Transactivation for each ligand (expressed as fold-induction over control cells) was plotted against the increase in luciferase units obtained in the presence of SMRT-VP16. ● ZK157202, ○ ZK161422, ■ vitamin D, □ ZK168289, ☆ ZK168492, ◆ ZK191732, ◇ ZK159222, ▲ ZK136607.

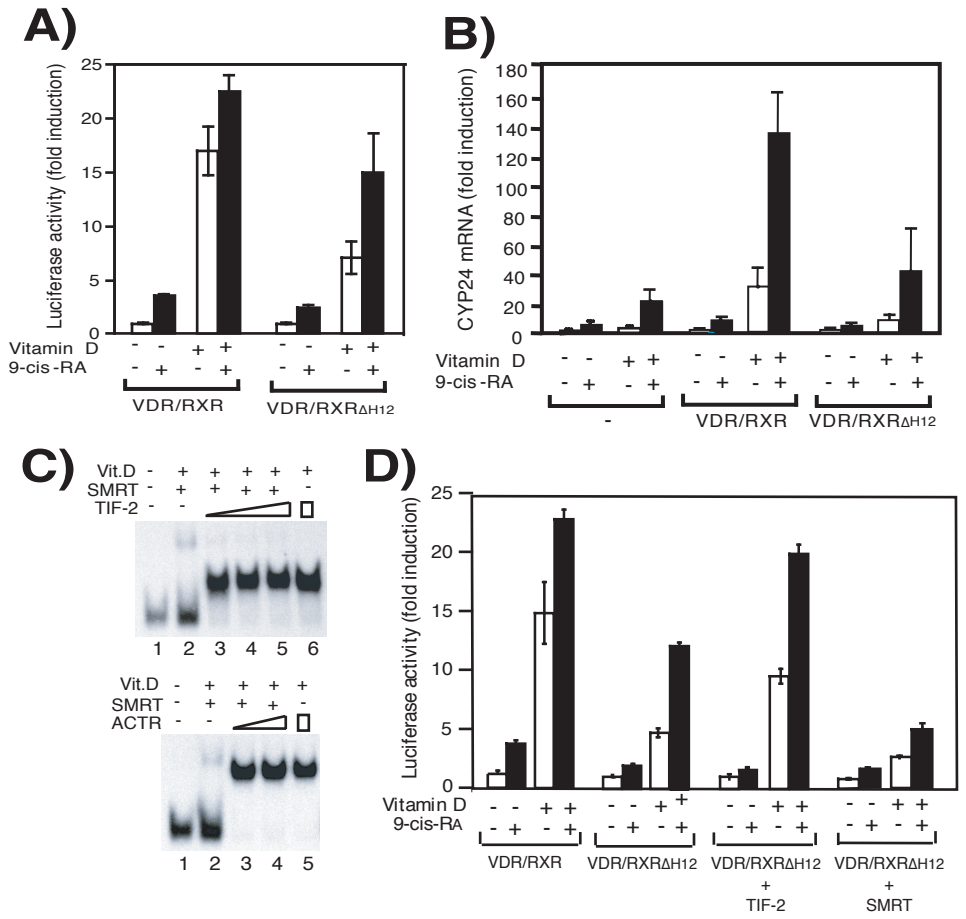

Figure 4. An increase in corepressors binding reduces the transcriptional response to vitamin D. (A) Luciferase activity in 293-T cells transfected with VDR/RXR or VDR/RXRΔH12 and the 4xVDRE reporter. Luciferase was measured in untreated cells and in cells treated with vitamin D and/or 9-*cis*-RA for 36 h. (B) *cyp*24 mRNA levels after 4 h of incubation with ligands in cells transfected with an empty vector, VDR/RXR or VDR/RXRΔH12. (C) Competition between SMRT and the p160 coactivators TIF-2 and ACTR for binding to VDR/RXR in response to vitamin D analyzed in gel retardation assays. (D) Luciferase activity in cells transfected with the receptors alone or in combination with expression vectors for TIF-2 or SMRT as indicated. Cells were treated as in (A).

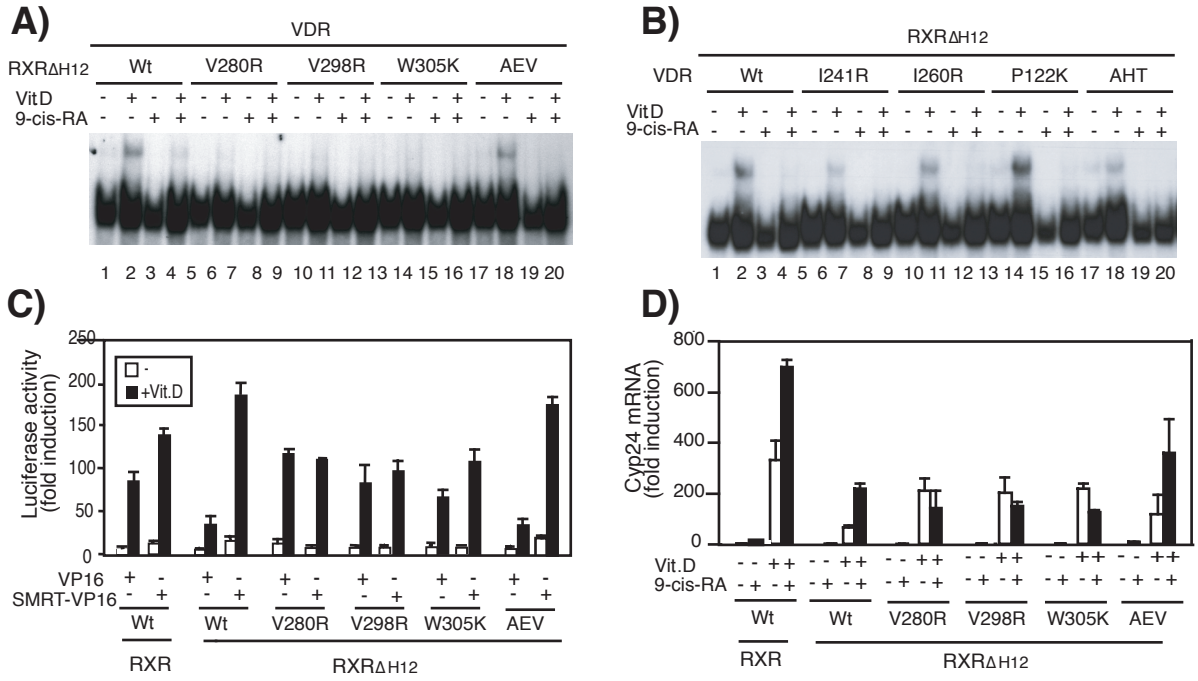

Figure 5. Mutation of the RXR corepressor binding surface abolishes SMRT interaction. (A and B) Gel retardation assays with the indicated point mutations in H3, 4 and 5 of the ligand binding domain belonging to the putative corepressor binding surface. AEV and the corresponding AHT mutation are in the CoR box in H1. (C) One hybrid assays 293-T cells with the mutants used in (A). Luciferase activity was measured in cells treated for 36 h with or without vitamin D. (D) *cyp*24 mRNA levels in cells transfected with the same mutant receptors and incubated with ligands for 4 h.

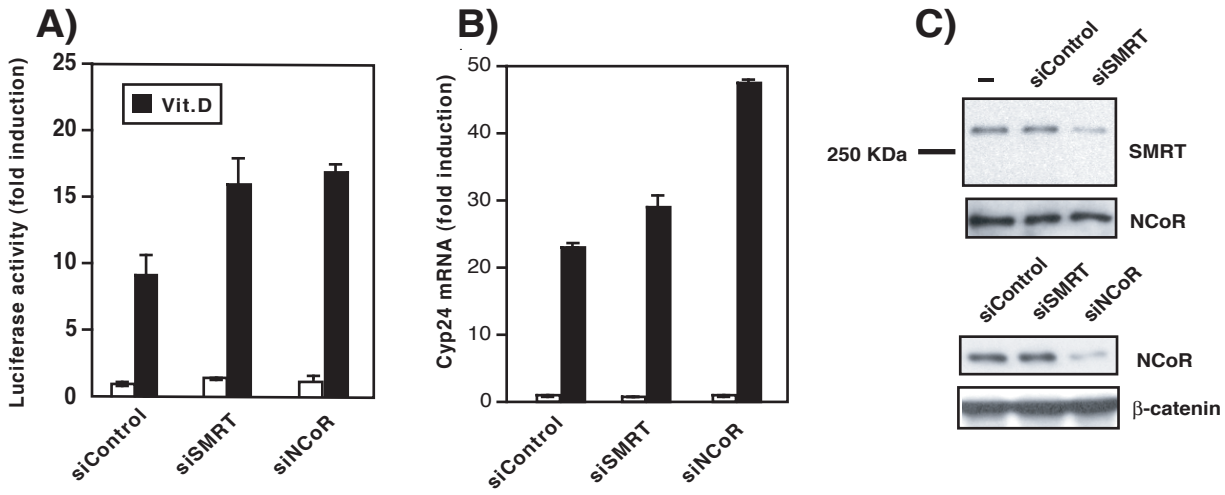

Figure 6. Corepressor knock-down increases vitamin D-dependent transcription. (A) Luciferase activity in 293-T cells cotransfected with the 4xVDRE reporter, VDR/RXR and control, SMRT or NCoR siRNAs. Reporter activity was determined after 48 h of incubation with vitamin D. (B) *cyp*24 transcripts in cells transfected with the receptors and siRNAs after 4 h of incubation in the presence and absence of vitamin D. (C) SMRT and NCoR levels determined by Western blot in cells transfected with the indicated siRNAs.

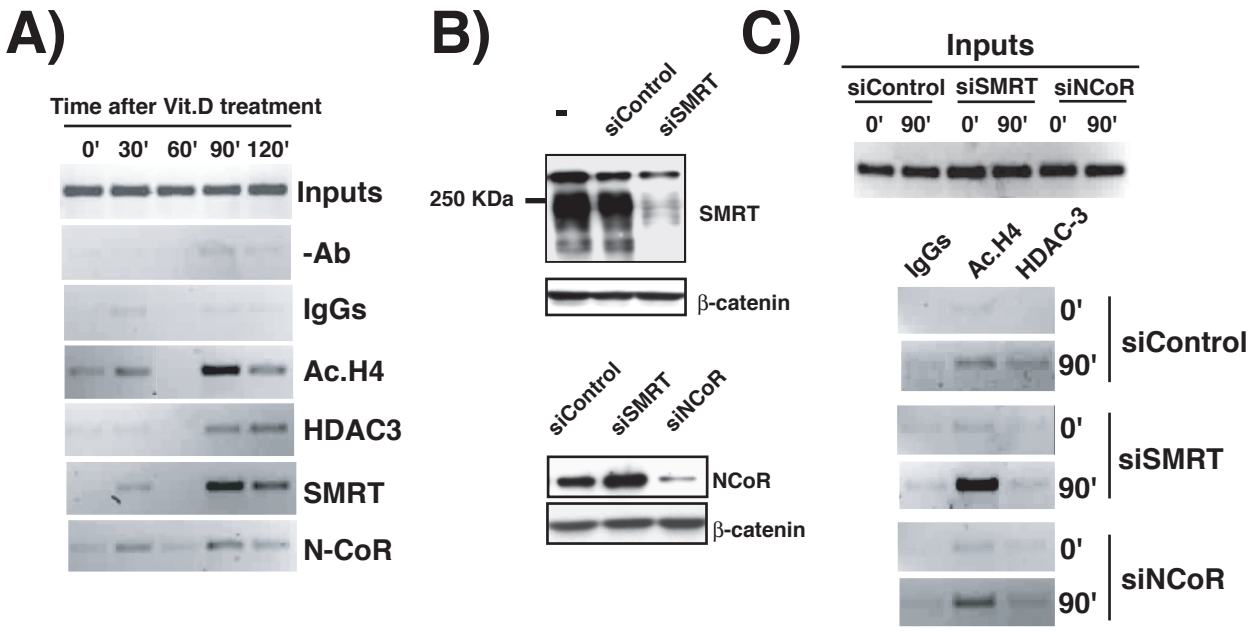

Figure 7. Recruitment of corepressors to the *cyp*24 promoter in MCF-7 cells that express endogenous receptors. (A) Binding of acetylated histone H4, HDAC3, SMRT and NCoR to the *cyp*24 promoter determined by ChIP assays at different time points after vitamin D treatment. (B) SMRT and NCoR expression in MCF-7 cells transfected with control, SMRT and NCoR siRNAs. (C) Enrichment in acetylated histone H4 and reduction of HDAC3 in the *cyp*24 promoter of MCF-7 cells transfected with siRNAs for the corepressors after 90 min of incubation with vitamin D.

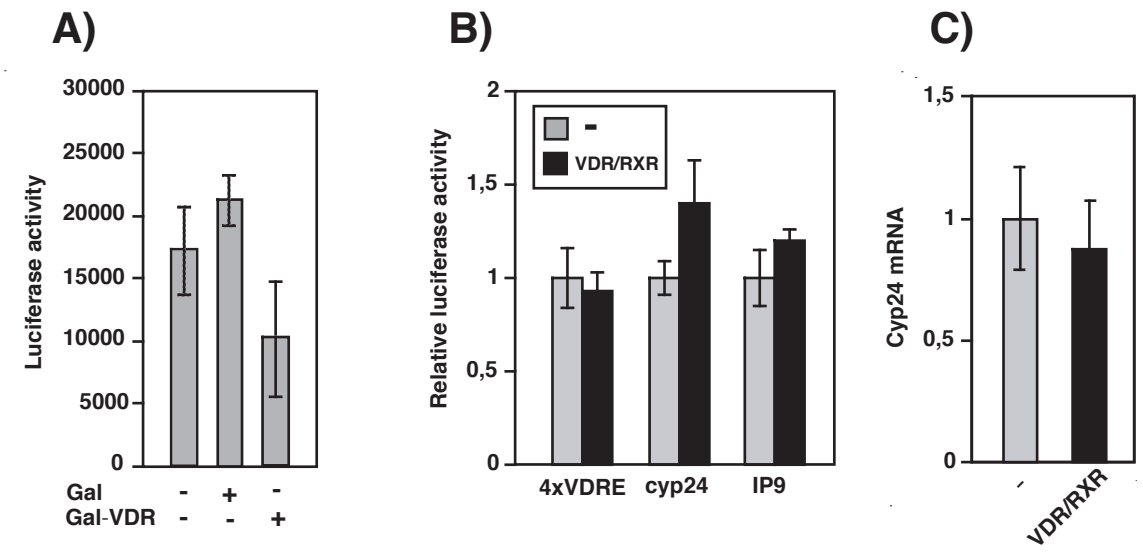

Figure S1. VDR lacks silencing activity. A) 293-T were transfected with an UAS reporter plasmid, the Gal DBD alone or the Gal DBD fused to VDR and luciferase activity was determined after 48 h. B) Cells were transfected with different VDRE-containing reporter plasmids: a construct containing 4 copies of a consensus DR3-type VDRE, a construct with a fragment of the VDRE-containing *cyp*24 promoter, and a construct with the inverted palindromic (IP9) VDRE found in the *c-fos* promoter. Luciferase activity was determined in cells transfected with an empty vector or with expression vector for VDR and RXR C) Cyp24 transcripts measured in cells transfected with the receptors or with the empty vector. Results and are expressed relative to the values obtained in cells transfected with the non coding vector.

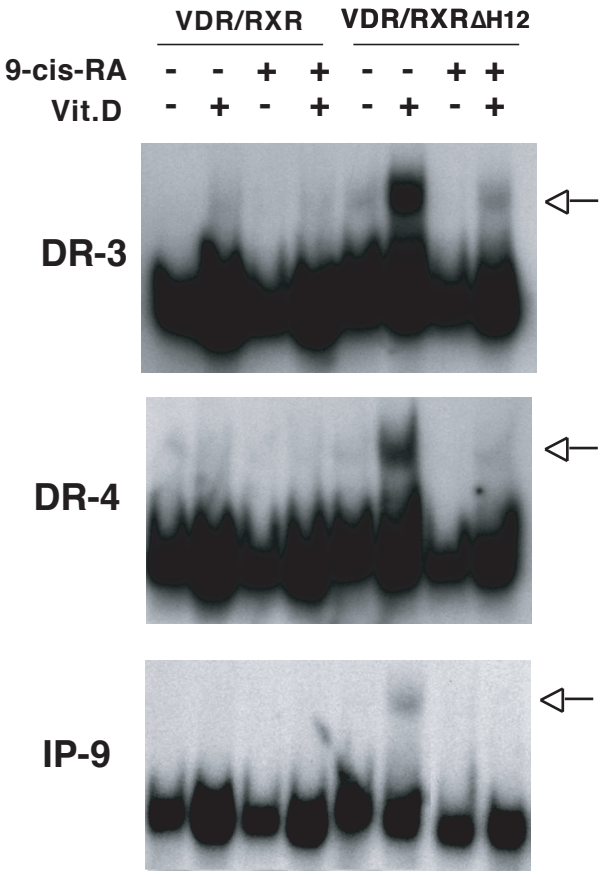

Figure S2. SMRT is recruited to various VDREs in a vitamin D-dependent manner. In vitro translated VDR/RXR and the truncated heterodimer VDR/RXRΔH12 were used for band-shift assays with SMRT and the oligonucleotides conforming the consensus DR3-type VDRE agctcAGGTCAaggAGGTCAg, the DR4-type VDRE agcttAGTTCAtgagAGTTCAg identified in the rat Pit-1 gene (1), and the IP9 VDRE agctTTGCCTgggtgaatgAGGACAg of the rat osteocalcin promoter (2). Vitamin D and 9-*cis*-RA were present in the assays as indicated. The mobility of the terciary complex containing the heterodimer and SMRT is indicated by arrows.

1) Toell, A., Polly, P., and Carlberg, C. (2000). All natural DR3-type vitamin D response elements show a similar functionality in vitro. Biochem J 352 Pt 2, 301-309

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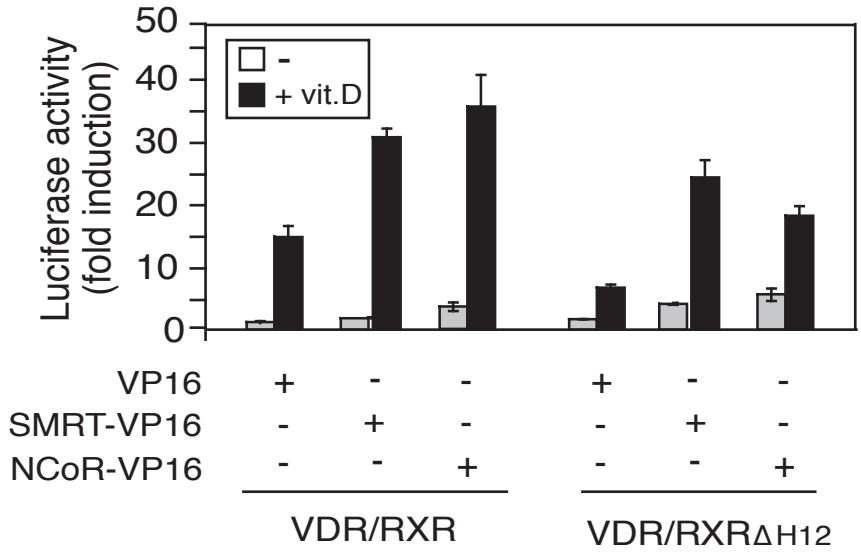

Figure S3. Both NCoR and SMRT interact "in vivo" with VDR/RXR heterodimers. "One hybrid" assays were performed in 293-T cells transfected with the 4xVDRE reporter and wild type or truncated VDR/RXR heterodimers. These constructs were cotransfected with the VP16 activation domain alone or fused to the C-terminal fragments of SMRT and NCoR that contain the receptor interacting domains. Luciferase activity was determined in control cells and in cells treated with 3 nM vitamin D for 36 h, as indicated.

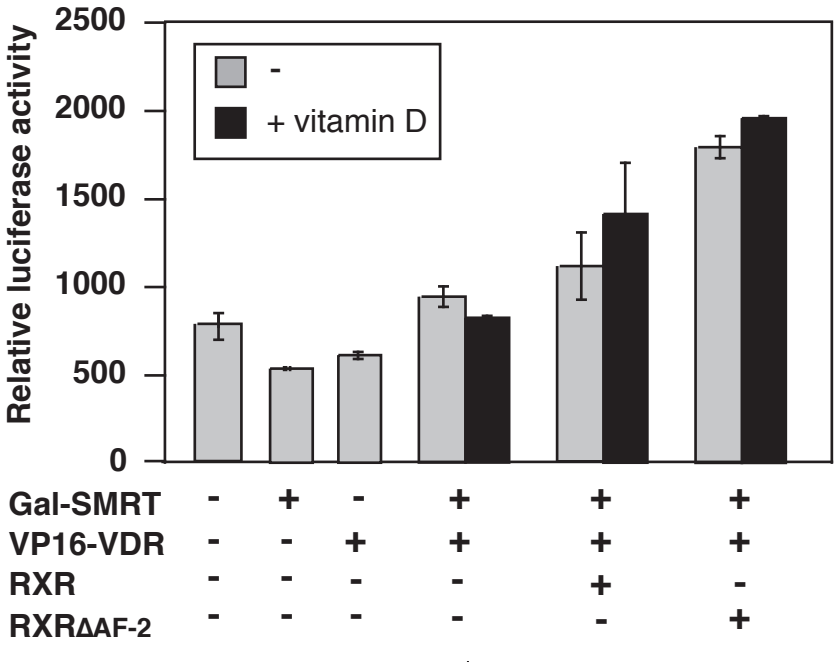

Figure S4. Vitamin D does not cause interaction of SMRT with VDR/RXR heterodimers in mammalian "two hybrid" assays. 293-T cells were transfected with an UAS reporter construct and the plasmids indicated. Luciferase activity was determined 36 h later in untreated cells and in cells treated with 3 nM vitamin D. In cells expressing RXRΔH12 a weak interaction of SMRT with VDR was observed, but vitamin D did not increase this association.

CHARACTERIZATION OF VITAMIN D RECEPTOR LIGANDS WITH CELL-SPECIFIC AND DISSOCIATED ACTIVITY

Ana I. Castillo, Ruth Sánchez Martínez, Ana M. Jiménez Lara, Andreas Steinmeyer, Ulrich Zügel y Ana Aranda

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Introducción al Capítulo 4

Además del mantenimiento de la homeostasis del calcio, la vitamina D desempeña otras importantes funciones entre las que se encuentran la modulación del sistema inmune y el control de la proliferación y diferenciación celular. Así los ligandos del VDR son importantes agentes terapéuticos para el tratamiento de la psoriasis, osteoporosis y el hiperparatiroidismo secundario, además de prometedoras sustancias antitumorales. En los últimos años se ha invertido un considerable esfuerzo en el desarrollo de compuestos que presenten esta acción terapéutica sin conllevar los peligrosos efectos hipercalcémicos propios de las dosis farmacológicas de VitD. En este trabajo hemos caracterizado varios derivados sintéticos de la VitD tanto *in vitro* como en experimentos con distintas líneas celulares. Estas sustancias presentan una afinidad por el receptor comparable a la de la VitD y unas actividades calcémicas en muchos casos sustancialmente menores que las del ligando natural. Hemos estudiado la conformación del receptor que provoca la unión de estos compuestos, el reclutamiento de coactivadores y sus efectos en transactivación y transrrepresión en las líneas celulares Hela y 293T. Para definir si estos análogos presentaban perfiles agonistas o antagonistas comparamos su comportamiento con el de otros previamente caracterizados, el superagonista ZK161422 y el antagonista parcial ZK159222. El compuesto ZK157202 produce un patrón de digestión con tripsina del receptor equivalente al observado con ZK161422. Además provoca un reclutamiento de coactivadores y una activación de la transcripción de construcciones reporteras que contienen VDREs de similar magnitud a ZK161422 e incluso mayor que la producida por la propia VitD. Este compuesto transrreprime la activación del promotor RARβ2 por ácido retinoico y la del gen de la colagenasa I por el TPA con una potencia similar a la del ligando natural del VDR. Por ello definimos este compuesto como un superagonista. Los compuestos ZK136607, ZK168492, ZK191732 y ZK168289, presentan un patrón en las digestiones parciales con proteasas similar al causado por el antagonista parcial ZK159222. Además no producen reclutamiento de coactivadores ni son efectivos en transactivación. Definimos entonces a estos compuestos como antagonistas parciales. Sin embargo, estas sustancias presentan distintos efectos dependiendo del tipo celular y de la clase de respuesta transcripcional analizada. Mientras que son incapaces de producir una activación transcripcional potente, si que transrreprimen con una potencia similar a la de la VitD. Estos "efectos disociados" hacen particularmente interesantes a estos análogos puesto que podrían modular procesos en los que la VitD juegue un papel represor sin afectar a otras respuestas transcripcionales donde podrían causar efectos indeseados. Por tanto, ya que estos ligandos de VDR presentan una afinidad por el receptor equiparable a la de la VitD y una actividad calcémica muy baja, podrían ser útiles herramientas farmacológicas en la prevención y tratamiento de enfermedades relacionadas con la señalización mediada por VDR.

La alumna ha participado en este trabajo en la puesta a punto y la realización de distintos ensayos de transfección transitoria para el estudio de la transactivación y la transrrepresión mediada por estos análogos de la VitD. También ha contribuido a la discusión de los resultados obtenidos.

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Characterization of Vitamin D Receptor Ligands with Cell-Specific and Dissociated Activity

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Although the main role of 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is to regulate calcium homeostasis, the valuable therapeutic applications of this compound have led to the search of new 1,25-$(OH)_2D_3$-vitamin D receptor (VDR) ligands with less side effects. In this work we have characterized seven 1,25-$(OH)_2D_3$ derivatives (ZK136607, ZK161422, ZK157202, ZK159222, ZK168492, ZK191732, and ZK168289). ZK157202 is an agonist that gives a pattern similar to that of 1,25-$(OH)_2D_3$ or ZK161422 in limited trypsin digestion assays, is able to recruit p160 and VDR-interacting protein 205 coactivators, is as potent as 1,25-$(OH)_2D_3$ to stimulate vitamin D response element-dependent transcription in HeLa cells, and acts as a superagonist in human embryonic kidney 293T cells. This compound is also more potent than the natural ligand to transrepress the activation of the retinoic acid receptor β2 promoter by retinoic acid and the response of the collagenase promoter to 4α-12-*O*-tetradecanoylphorbol 13-acetate. ZK136607, ZK168492, ZK191732, and ZK168289 have a profile similar to that of the partial antagonist ZK159222. They induce an antagonistic-type proteolytic pattern, do not recruit classical coactivators, and have little transactivation potency. However, they act in a cell context-dependent manner because they lack activity in HeLa cells while presenting some agonistic activity in human embryonic kidney 293T cells, or *vice versa*. Furthermore, some of these compounds have a dissociated activity: they cannot transactivate but they are as potent as 1,25-$(OH)_2D_3$ in transrepression assays. Together our results demonstrate the existence of novel VDR ligands with variable biological functions and dissociated activity. They should represent useful tools for studying VDR function and could have therapeutic utility. (*Molecular Endocrinology* 20: 3093–3104, 2006)

MOST OF THE biological actions of 1α, 25-dihydroxyvitamin D_3 [calcitriol, 1,25-$(OH)_2D_3$] are mediated by the receptor VDR (vitamin D receptor), a member of the nuclear receptor superfamily of ligand-dependent transcription factors (1). VDR acts preferentially as a heterodimer with RXR (retinoid X receptor) through binding to specific DNA sequences located at regulatory regions of target genes, referred to as vitamin D response elements (VDREs), normally composed of two copies of the consensus AGGTCA motif arranged as a direct repeat spaced by three nucleotides (DR3). The nuclear receptors exhibit a modular structure with several functional domains. The ligand binding domain (LBD) contains the COOH-terminal activation function (AF)-2 motif responsible for ligand-dependent transcriptional activation. In this process, a critical step in nuclear receptor signaling is the specific ligand-triggered induction of a conformational change within the LBD (2). This conformational change results in ordered recruitment of coactivators. The p160 coactivators [steroid receptor coactivator (SRC)-1/nuclear coactivator 1, transcriptional intermediary factor (TIF)2 or p300/cAMP response element binding protein-binding protein/activator for thyroid hormone and retinoid receptors (ACTR)] have intrinsic histone acetyltransferase activity and recruit additional histone acetyltransferase and histone methyltranferase enzymes that alter chromatin structure and modulate gene transcription (3, 4). The receptors also recruit ATP-dependent chromatin remodeling complexes, in the case of VDR through direct interaction with WSTF, a component of the WINAC complex (5). In addition, the subunit VDR-interacting protein (DRIP)205/thyroid hormone receptor-associated protein 220 (6) of the DRIP/ thyroid hormone receptor-associated protein/Mediator multiprotein complex is also recruited to the core AF-2 receptor region in response to ligand binding. It has been suggested that this complex

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Abbreviations: ACTR, Activator for thyroid hormone and retinoid receptors; AF-2, activation function 2; CAT, chloramphenicol acetyltransferase; c1LPD, conformation 1 of the limited limited protease digestion; DRIP, VDR-interacting protein; GHF-1, GH transcription factor-1; GST, glutathione-*S*-transferase; HEK, human embryonic kidney; LBD, ligand-binding domain; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; RXR, retinoid X receptor; SRC, steroid receptor coactivator; TIF, transcriptional intermediary factor; TPA, 4α-12-*O*-tetradecanoylphorbol 13-acetate; VDR, vitamin D receptor; VDRE, vitamin D response element; VDRM, VDR modulator.

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could recruit the holoenzyme of the RNA polymerase II to the target promoter (7).

In contrast to positively regulated genes, the mechanism by which nuclear receptors control the transcription of negatively regulated genes is less well understood. Proposed mechanisms include: competition of nuclear receptors with other transcription factor binding sites (8–10); receptor binding to the so called negative regulatory elements in which the receptor interacts with other factors and recruits corepressors in a ligand-dependent manner (11); direct interactions of nuclear receptors with transcription factors such as the jun component of the AP-1 complex, precluding a productive interaction with coactivators or basal factors; and competition for limiting amounts of transcriptional cofactors (1).

It is widely accepted that the fundamental role of 1,25-$(OH)_2D_3$ is to regulate calcium homeostasis (12). However, many other biological functions of this vitamin have been reported. Indeed, 1,25-$(OH)_2D_3$ plays an important role in promoting cellular differentiation, and in inhibiting the growth of several primary and cultured cancer cell types, including T cell leukemias, breast, prostate, and colon. It has also been proposed that 1,25-$(OH)_2D_3$ and its synthetic analogs could be useful in renal failure, vitamin D-dependent rickets type I, osteoporosis, psoriasis, and certain autoimmune disorders such as multiple sclerosis or type 1 diabetes mellitus, although clinical data proving their efficacy are not yet available (13, 14).

Although these findings suggest new therapeutic possibilities for 1,25-$(OH)_2D_3$, deleterious side effects such as hypercalcemia and soft tissue calcification prevent the use of 1,25-$(OH)_2D_3$ as a therapeutic agent. Therefore, a great deal of effort is being made to develop new 1,25-$(OH)_2D_3$ analogs to dissociate immunosuppressive/growth inhibitory/differentiation properties and calcemic effects (15–18). Very recently, novel noncalcemic, tissue selective, nonsecosteroidal vitamin D receptor modulators (VDRMs) with improved therapeutic indices have been obtained and characterized (19).

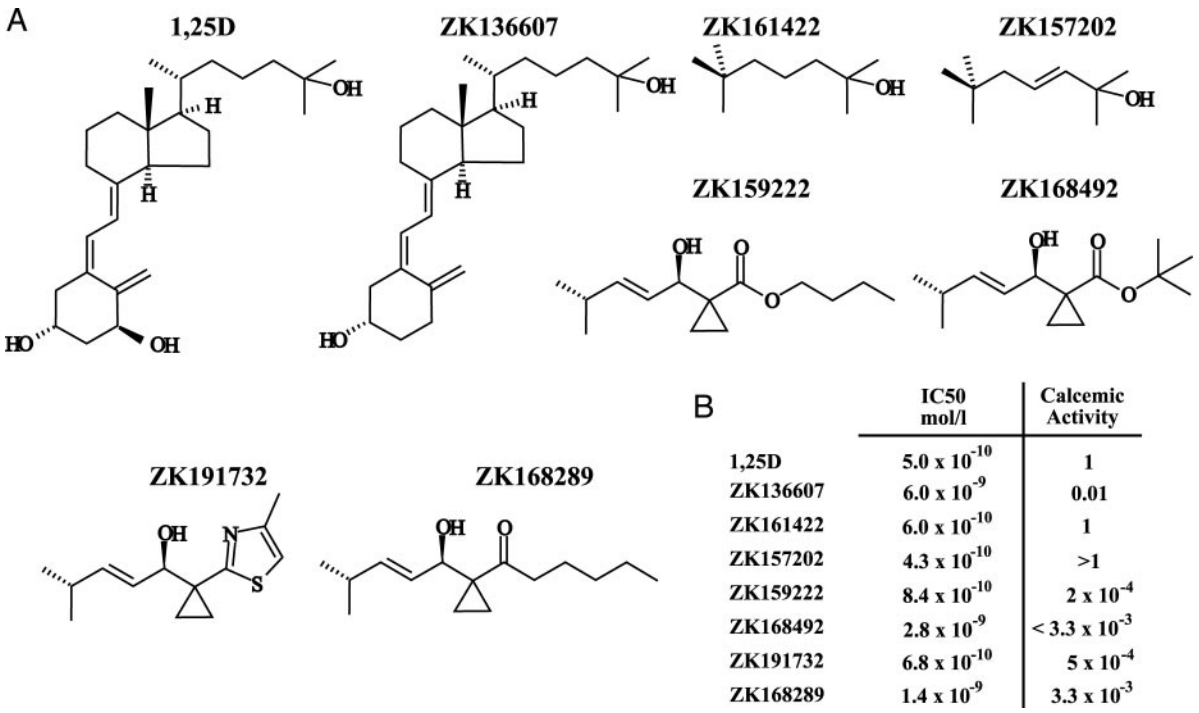
In this study, we have compared the biological actions of seven 1,25-$(OH)_2D_3$ derivatives synthesized by Schering AG (ZK136607, ZK161422, ZK157202, ZK159222, ZK168492, ZK191732, ZK168289). We have analyzed them for their agonistic and antagonistic profile *in vitro* by monitoring the consequences of ligand binding on receptor conformation and on the recruitment of coactivator complexes. We have also studied the effects of these compounds on transactivation and transrepression of target gene promoters in HeLa and human embryonic kidney (HEK) 293T cellular systems. In our study, ZK161422, described as an agonist (20) and ZK159222, described as an antagonist with residual agonistic activity (21), were chosen to compare with the effects promoted by the other compounds and by the natural ligand. Our results show that ZK157202 as well as ZK161422 have a clear agonist profile and that they are even more potent than 1,25-$(OH)_2D_3$ in both transactivation and transrepression. However, other compounds have a profile similar to that of the ZK159222 partial antagonist. They induce an antagonistic-type proteolytic pattern, they are unable to stimulate the recruitment of classical coactivators, and they have little transactivation potency. However, the agonistic effect appears to depend on the cell context, and some of these compounds have a dissociative activity: they cannot transactivate but they are as potent as 1,25-$(OH)_2D_3$ in transrepression assays. These dissociated 1,25-$(OH)_2D_3$ analogs, here identified, are potential pharmacological tools in the treatment and prevention of diseases in which VDRs play a role.

RESULTS

Effect of 1,25-$(OH)_2D_3$ Analogs on Receptor Conformation

The chemical structure, binding affinities and calcemic activity of the 1,25-$(OH)_2D_3$ analogs used are shown in Fig. 1. In an *in vitro* binding assay, unlabeled ZK161422, ZK157202, ZK159222, and ZK191732 bound to VDR with a potency similar to that of 1,25-$(OH)_2D_3$, whereas the other three ligands, ZK136607, ZK168492, and ZK168289, bound the receptor with 1 order of magnitude lower than 1,25-$(OH)_2D_3$. On the other hand, the calcemic activity of ZK161422 was similar to that of the natural ligand, and that of ZK157202 was ever higher, whereas the remaining VDR ligands presented a markedly reduced calcemic activity, measured both as urine calcium levels (Fig. 1) and serum calcium levels (data not shown).

The ability of a ligand to induce transactivation of the nuclear receptor can be described as a combination of affinity, kinetics, and effectiveness at producing an optimal protein conformation that facilitates the interaction with coactivator proteins, which consequently results in stimulation of transcriptional activity through various additional protein-protein interactions. We performed limited protease digestion assays, in which the interaction of a nuclear receptor with its ligand protects the LBD against protease digestion, as a method for characterizing functional VDR conformations. In this assay, VDR was subjected to limited proteolysis with trypsin in the presence of a saturating concentration of 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs (10 μM). 1,25-$(OH)_2D_3$ generates a predominant 28-kDa fragment and a minor 23-kDa fragment represented in Fig. 2A as c1LPD or c3LPD, respectively (conformations 1 and 3 of the limited protease digestion). The agonist ZK161422, as well as the ZK157202 compound, induces the same proteolytic pattern as 1,25-$(OH)_2D_3$. However, the antagonist ZK159222, as well as ZK136607, ZK168492, ZK191732, and ZK168289, generates an additional fragment at 25 kDa (designated c2LPD in Fig. 2A). c1LPD, c2LPD, and c3LPD contain major parts of the LBD and its carboxyl-



	IC50 mol/l	Calcemic Activity
1,25D	5.0×10^{-10}	1
ZK136607	6.0×10^{-9}	0.01
ZK161422	6.0×10^{-10}	1
ZK157202	4.3×10^{-10}	>1
ZK159222	8.4×10^{-10}	2×10^{-4}
ZK168492	2.8×10^{-9}	$< 3.3 \times 10^{-3}$
ZK191732	6.8×10^{-10}	5×10^{-4}
ZK168289	1.4×10^{-9}	3.3×10^{-3}

Fig. 1. Structure and Characteristics of 1,25-$(OH)_2D_3$ Analogs

A, Chemical structures of 1,25-$(OH)_2D_3$ and the ZK compounds. Only the side chains that are different from that of the natural ligand are depicted for some of the analogs. B, VDR ligand binding and calcemic activities. VDR ligand binding is expressed as IC_{50} [concentration of ligand required to inhibit the binding of labeled 1,25-$(OH)_2D_3$ by 50%]. For the calcemic activities, the value 1, obtained with 0.03 μg/kg/d of 1,25-$(OH)_2D_3$, is used as a reference. The doses that were equipotent with this concentration of 1,25-$(OH)_2D_3$ are given for the ZK compounds. The maximal concentration used was 10 μg/kg/d. 1,25-$(OH)_2D_3$ is shown as 1,25D.

terminal truncations including from the trypsin-cutting site after arginine 173 to either the carboxy terminus at position 427 (c1LPD), to arginine 402 (c2LPD), or to arginine 391 (c3LPD) (21). The 28- and 23-kDa fragments reported here are thought to be the same previously referred to as the 34- and 30-kDa fragments (22). The 28-kDa (21) or 34-kDa (22) fragments contain a 19-residue portion of the hinge region and the entire LBD. The other shorter fragments have the same N terminus (after the arginine 173 trypsin-cutting site), but result from further trypsinization near the C terminus. An increase in the intensity of these fragments could be explained as a failure of the ligand to coordinate the active closed conformation of the helices 10–12 of the LBD, leaving them more susceptible to proteolytic cleavage. It has been suggested that these shorter fragments are indicative of a transcriptionally inactive state (22). In agreement with others (23), our results suggest that ZK157202 could be a potential agonist because the conformational change in VDR induced by this analog is very similar to that induced by 1,25-$(OH)_2D_3$ and the ZK161422 agonist (in which the 28- or 30-kDa fragments are predominant). In contrast, the proteolytic pattern of VDR observed in the presence of ZK136607, ZK168492, ZK191732, and ZK168289 is similar to that resulting from binding of the antagonist ZK159222 to the VDR. ZK191732 has already been demonstrated to behave as an antagonist of 1,25-$(OH)_2D_3$-induced differentiation of Caco-2 cells (24).

Analysis of VDR-Coactivator Interactions in the Presence of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ Analogs

As an additional approach, we have analyzed the agonistic or antagonistic potential of the 1,25-$(OH)_2D_3$ analogs based on their ability to induce an interaction with coactivator proteins. For this purpose, we have performed glutathione-*S*-transferase (GST) pull-down and supershift assays. GST pull-down assays were performed with bacterially produced GST-TIF2 (624–1287), GST-ACTR (621–821), GST-SRC1 (570–780), and GST DRIP205 (1770–2556) (fusion proteins containing the coactivators nuclear receptor interaction domain) and *in vitro* translated ^{35}S-labeled VDR in the presence of 100 nM 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs (Fig. 2B). Supershift assays were performed with the same coactivator proteins used in the pull-down assays, *in vitro* translated VDR-RXR heterodimers, and a consensus DR3-type VDRE in the presence of a saturating concentration of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs (1 μM) (Fig. 2C).

In both assays, 1,25-$(OH)_2D_3$, the agonist ZK161422, and the ZK157202 compound were able to

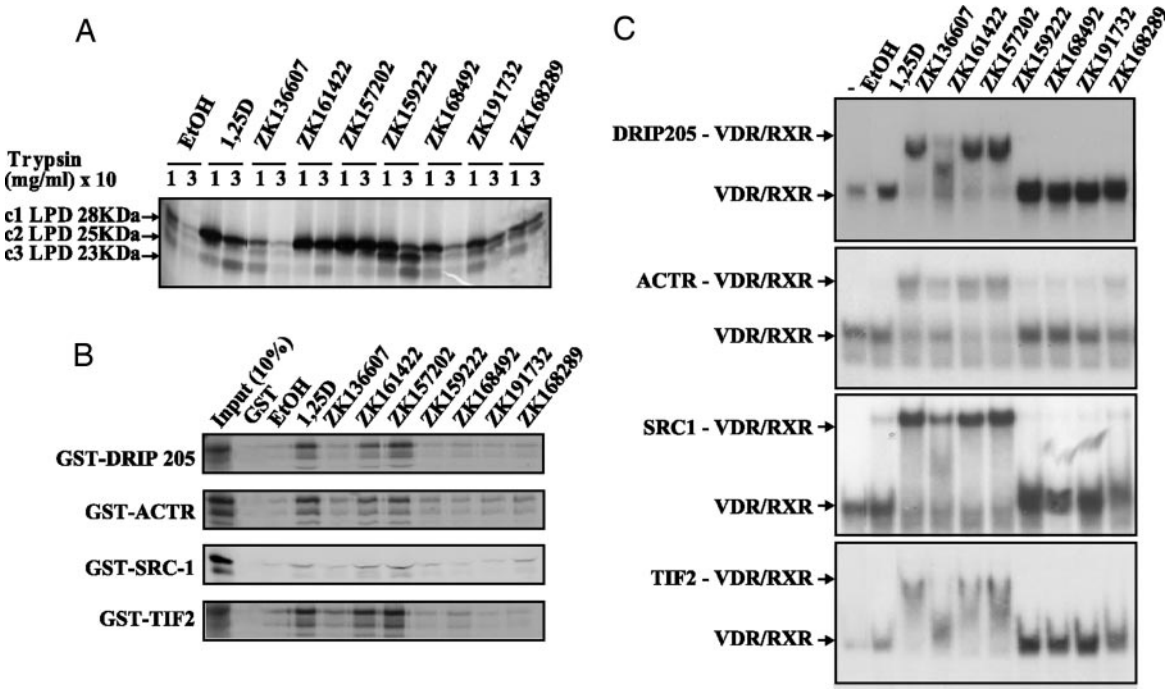


Fig. 2. Effect of 1,25-$(OH)_2D_3$ Analogs on VDR Conformation and Coactivator Recruitment

A, Limited protease digestion assay. *In vitro* translated [^{35}S]VDR preincubated with EtOH (as a negative control), 10 μM of 1,25-$(OH)_2D_3$ (as a positive control), and 10 μM 1,25-$(OH)_2D_3$ analogs (ZK compounds) were digested with two concentrations of trypsin (10 or 30 μg/ml). B, Pull-down assays performed with *in vitro* translated [^{35}S]VDR and the indicated GST coactivators. VDR was incubated with EtOH (as a negative control), 100 nM 1,25-$(OH)_2D_3$ (as a positive control), and 100 nM 1,25-$(OH)_2D_3$ analogs. C, Gel retardation assays were performed with *in vitro* translated VDR/RXR heterodimers that were preincubated in the presence of bacterially expressed GST coactivators with EtOH (as a negative control), 1 μM 1,25-$(OH)_2D_3$ (as a positive control), 1 μM ZK ligands, and the ^{32}P-labeled DR3-type VDRE. 1,25-$(OH)_2D_3$ is shown as 1,25D.

induce the interaction of VDR with the coactivators. In contrast, ZK159222, ZK168492, ZK191732, and ZK168289 were not able to promote significant coactivator recruitment, either in solution or in a complex with RXR on DNA. These results correlate with the conformational change observed in the limited protease digestion assays described above.

Interestingly, ZK136607 was not able to induce interaction of VDR with the coactivators in solution but showed a slight capacity to promote VDR-coactivator interaction in a complex with RXR on DNA. Although this analog stabilized the VDR conformation c2LPD, it is possible that in the presence of RXR and DNA, ZK136607 is able to generate a more agonistic conformation in VDR, which allows a partial recruitment of coactivators by this receptor. This ligand also shows a reduced affinity by VDR as compared with 1,25-$(OH)_2D_3$ in binding assays (see Fig. 1), but the concentration used in the supershift assays is high enough to saturate the receptor; therefore, its reduced capacity of coactivator recruitment cannot be secondary to its decreased binding affinity.

Effect of VDR Ligands on Transactivation Assays

We performed transient transfections with a VDRE-containing heterologous promoter (4×VDRE TK-Luc) in human HEK 293T (Fig. 3A) and HeLa cells (Fig. 3B), transfected with receptors in the presence of graded concentrations of 1,25-$(OH)_2D_3$ or ZK compounds. 1,25-$(OH)_2D_3$ induced reporter activity in a typical dose-dependent manner, achieving the maximal effect (20-fold induction) at 10 nM in HEK 293T cells or at 100 nM in HeLa cells. As expected, the agonist ZK161422 was as potent as 1,25-$(OH)_2D_3$ to transactivate the DR3-containing plasmid. In addition, at low doses, ZK157202 was even more potent than 1,25-$(OH)_2D_3$. This superagonistic effect was more marked in HEK 293T cells, achieving the maximal action at 1 nM. In contrast, the other compounds showed a null or partial agonist activity. As expected, ZK159222 considered as a partial antagonist, only weakly activated the promoter at the highest dose used. ZK136607 showed null (HEK 293T cells) or low activity (Hela cells), and ZK168492 also showed more activity in HeLa than in HEK 293 cells. In contrast, in HEK 293T, but not in HeLa cells, ZK191732 and ZK168289 activated the promoter although always with less potency than 1,25-$(OH)_2D_3$. Although a weaker increase of reporter activity was found in HeLa cells that were not transfected with receptors, the transactivation profile obtained with the different compounds was similar to that shown in Fig. 3B (data not shown), demonstrating

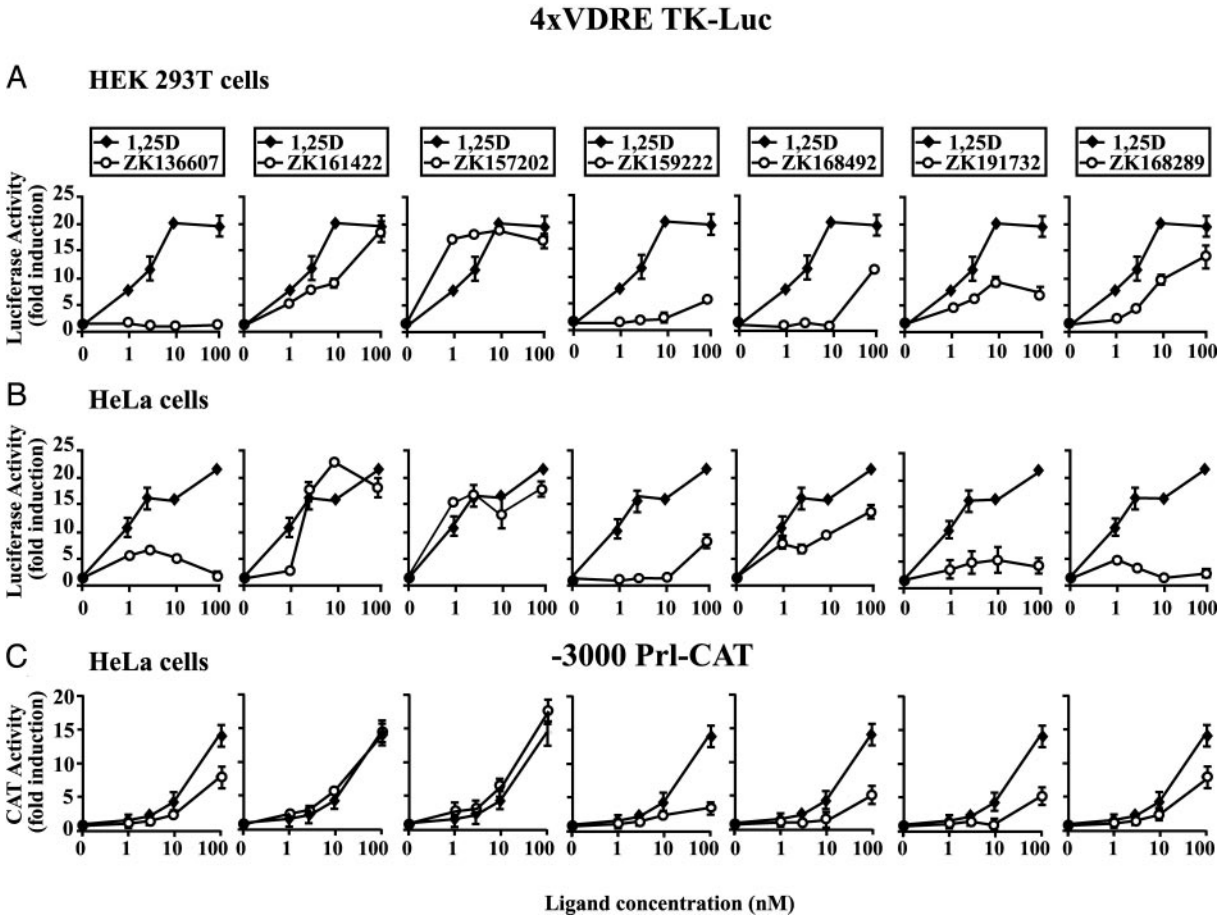


Fig. 3. Influence of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ Analogs on Transactivation

A, HEK 293T cells were cotransfected with 40 ng of 4×VDRE TK-Luc and 12.5 ng of VDR and RXR. B, HeLa cells were cotransfected with 500 ng of 4×VDRE TK-Luc and 80 ng of VDR and RXR. C, HeLa cells were cotransfected with 2.5 μg of −3000 Prl-CAT and expression vectors for the pituitary transcription factor GHF-1/Pit-1 (0.4 μg) and VDR (2.5 μg). Cells were treated for 48 h with graded concentrations of 1,25-$(OH)_2D_3$ (1,25D) and ZK compounds. CAT or Luc activity is expressed as fold induction over the values obtained in EtOH-treated control cells.

that the results obtained are applicable to a situation in which cells express low endogenous receptor levels.

We also performed transient transfections with a prolactin promoter construct (−3000 Prl-CAT), which contains a VDRE (25), as a model to analyze the role of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs on a natural complex promoter (Fig. 3C). Although HeLa cells, a cervical carcinoma cell line, do not express endogenous prolactin, expression of the pituitary-specific transcription factor GHF-1 (GH transcription factor-1)/Pit-1 and VDR causes a marked prolactin promoter stimulation by 1,25-$(OH)_2D_3$ that allows the analysis of transcriptional regulation in this heterologous cell system (25, 26). The dose of VDR ligands required to obtain the maximal activity was higher in the case of the prolactin promoter. Thus, the treatment of HeLa cells with 10 nM 1,25-$(OH)_2D_3$ strongly increased the heterologous promoter (Fig. 3B), whereas this concentration only promoted approximately one third of the maximal prolactin promoter activation (Fig. 3C). This discrepancy could be explained because the amount of transfected VDR used to activate the prolactin promoter in response to 1,25-$(OH)_2D_3$ was 3 times higher than that used to activate the heterologous promoter. ZK157202 was again more potent than 1,25-$(OH)_2D_3$, and the activity of ZK161422, ZK159222, and ZK168492 was similar to that found with the 4×VDRE TK-Luc construct in this cell type. Interestingly, ZK136607, ZK191732, and ZK168289 showed more agonistic potency in the context of the prolactin promoter.

The ability of compounds ZK136607, ZK159222, ZK168492, and ZK191732 to antagonize 1,25-$(OH)_2D_3$-dependent transactivation was tested in HEK 293T cells (Fig. 4, *upper panel*) and that of compounds ZK136607, ZK159222, ZK191732, and ZK168289 in Hela cells (Fig. 4, *lower panel*). In these assays the cells, transfected with the 4×VDRE TK-Luc plasmid, were treated with a maximal concentration (1 μM) of the compounds in the absence and presence of 10 nM of 1,25-$(OH)_2D_3$. In HeLa cells, ZK191732 showed the most significant antagonistic effect, reducing 1,25-

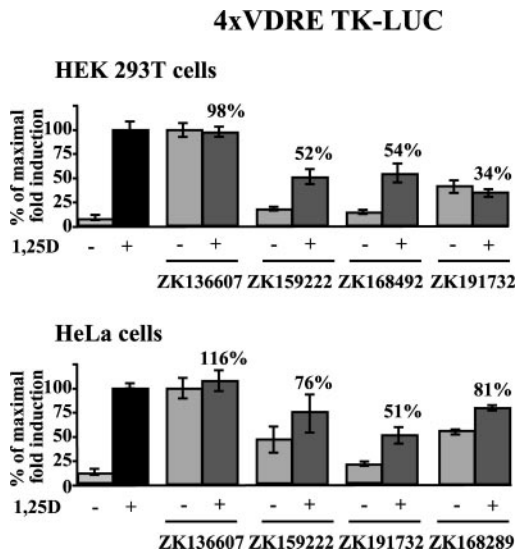


Fig. 4. Antagonism of 1,25-$(OH)_2D_3$-Dependent Transactivation

HEK 293T cells (*upper panel*) and HeLa cells (*lower panel*) were transfected with the 4×VDRE TK-Luc reporter as in Fig. 3. Cells were treated with 1 μM of the ZK analogs indicated in the absence and presence of 10 nM 1,25-$(OH)_2D_3$ (1,25D), and luciferase activity was determined after 48 h. Data are expressed relative to the maximal induction obtained in cells treated with 1,25-$(OH)_2D_3$ alone that were considered as 100%.

$(OH)_2D_3$-dependent promoter activation by 51%, whereas ZK159222 and ZK168289 alone provided approximately 50–60% of the maximal induction of reporter activity, and only a weak antagonistic effect was observed after 1,25-$(OH)_2D_3$ cotreatment. ZK136607, which had a null or very weak agonistic effect at lower doses (see Fig. 3), activated strongly reporter activity at 1 μM and consequently no antagonist effect was observed in the presence of 1,25-$(OH)_2D_3$ in either HEK 293T or HeLa cells. As occurred with the agonistic effects, antagonist potency was also cell context dependent. Thus, ZK159222 and ZK191732 showed more antagonistic activity in HEK 293T cells than in HeLa cells. ZK191732 was the most antagonistic compound in HEK 293T cells, and the combination of this compound with 1,25-$(OH)_2D_3$ resulted in only 34% of maximal induction.

Dissociated Activity of VDR Ligands

We have used the retinoic acid receptor (RAR)β2 (R140-Luc) and collagenase (−73Col-Luc) promoters as models to study transrepression by VDR ligands (Figs. 5 and 6). We have previously shown that 1,25-$(OH)_2D_3$ exerts a repressive effect on retinoic acid (RA)-dependent transactivation of the RARβ2 promoter. Competition for DNA binding site and titration of coactivator proteins are mechanisms suggested to explain this repression (9, 10). In HEK 293T (Fig. 5A) or Hela cells (Fig. 5B), as expected, the agonist ZK161422 and the potential superagonist ZK157202 were able to transrepress even more strongly than 1,25-$(OH)_2D_3$. It was expected that the VDR ligands promoting an incorrect positioning of the AF-2 surface, which does not allow the interaction of VDR with coactivators, should not transrepress RA-dependent transactivation. Surprisingly, the compounds ZK136607, ZK159222, ZK168492, ZK191732, and ZK168289, with null or weak agonistic activity, were also able to promote transrepression. Furthermore, inhibition was more evident at low doses, and some of these compounds with a low transactivation capacity were even more potent than 1,25-$(OH)_2D_3$ to transrepress the RARβ2 promoter. Cell-specific differences in the potency of the VDR ligands to inhibit the retinoic acid (RA) response were also observed. For instance, ZK136607 was more effective than the natural ligand in HeLa cells, whereas it was less potent in HEK 293T cells.

We also analyzed the transrepression exerted by the 1,25-$(OH)_2D_3$ analogs on 4α-12-*O*-tetradecanoylphorbol 13-acetate (TPA)-dependent transactivation of the collagenase promoter in HEK 293T (Fig. 6A) and HeLa cells (Fig. 6B). The results obtained with the −73Col-Luc construct were very similar to those obtained with the RARβ2 promoter in HEK 293T cells: ZK161422 and ZK157202 were more active than 1,25-$(OH)_2D_3$, and all the compounds with an antagonistic profile were able to transrepress the effect of TPA. However, in Hela cells, ZK136607, ZK191732, and ZK168289 transrepressed weakly in comparison with 1,25-$(OH)_2D_3$ and, paradoxically, only at low doses. Thus, the dissociated effect on transrepression *vs.* transactivation also appears to depend on the cellular context.

DISCUSSION

In this study, several 1,25-$(OH)_2D_3$ derivatives have been analyzed for their agonistic or antagonistic potential. Results obtained from the limited protease assays suggest that ZK136607, ZK168492, ZK191732, and ZK168289 could have low agonistic activity because they stabilize the VDR conformation c2LPD, which keeps helix 12 in a displaced position that does not allow an interaction of VDR with coactivators. The latter mechanism, which is based on an incorrect positioning and blocking of the AF-2 domain, has also been suggested for antagonists of other members of the nuclear hormone receptor superfamily, such as the estrogen receptor (27). In contrast with these compounds, ZK157202 appears to stabilize c1LPD even more than 1,25-$(OH)_2D_3$. In the presence of this compound, the shorter fragment c3LPD was only observed when a high dose of trypsin was used. This result suggests that this compound could be a potential superagonist. This agrees with the concept that superagonists are able to stabilize the agonistic conformation for a much longer time than the natural agonist (28).

Although ligand binding increases formation of c1-, c2-, and c3LPD, these bands are also detected in the

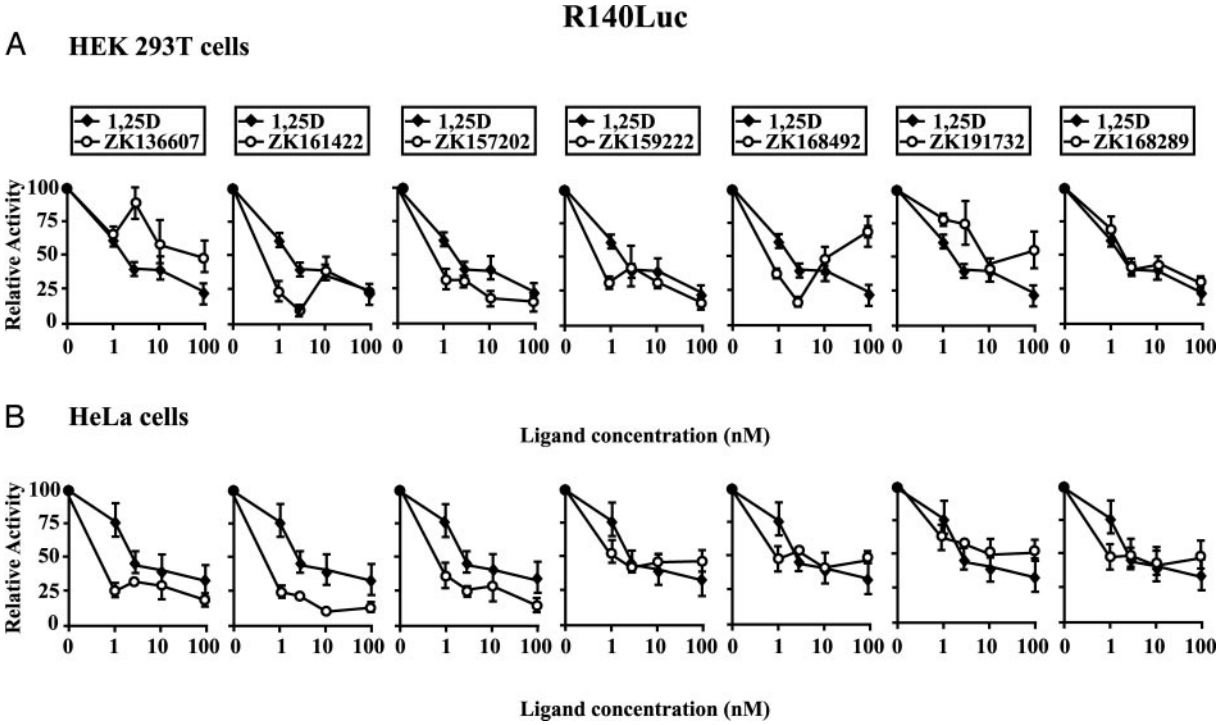


Fig. 5. Influence of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ Analogs on RA-Dependent Transactivation of the RARβ2 Promoter

A, HEK 293T cells were cotransfected with 200 ng of the RARβ2 promoter (R140-Luc), and expression vectors for VDR (12.5 ng), RXR (12.5 ng), and RAR (2.5 ng). B, HeLa cells were cotransfected with 500 ng of the RARβ2 promoter and VDR (80 ng), RXR (80 ng), and RAR (16 ng). After transfection the cells were treated for 48 h with 1 μM RA alone or in combination with increasing concentrations of 1,25-$(OH)_2D_3$ (1,25D) or ZK compounds, as indicated. Results are shown as a percentage of the luciferase activity obtained in the cells treated with RA alone.

absence of ligand, in agreement with the idea that different VDR conformations exist (29). The production of c3LPD has been recently linked to a putative VDR alternative binding pocket that is proposed to be kinetically favored by vitamin D sterols (30). Interestingly, it has been proposed that occupation by an appropriately shaped ligand can lead to the onset of either rapid or genomic VDR-mediated responses (29).

In all crystal structures of VDR bound to agonist ligands, a single conformation of the complex is observed: the position and conformation of the activation helix 12 is strictly maintained (31–33). In this agonistic conformation, precise positioning of helix 12 via the H397-F422 interaction creates a distance of 19Å between the negatively charged E420 on the surface of helix 12 and the positively charged K246 on the surface of helix 3. This charge clamp structure is essential for contacting the LXXLL motif of the NR interacting box of coactivator proteins. In fact, only ZK161422 and ZK157202, but not the other compounds, were able to induce, as 1,25-$(OH)_2D_3$ does, coactivator recruitment in solution and in the presence of RXR and DNA.

On the other hand, it has been suggested that the partial antagonist ZK159222 or the full antagonist ZK168281 adopt an structure in which the last four carbon atoms extend toward helices 3 and 12 and steric contacts are observed with A231 (helix 3) and V418 (helix 12), suggesting that most likely the activation helix will not be optimally positioned (31, 32). Molecular dynamics (MD) simulations of VDR's LBD have also demonstrated that the extended side chain of both antagonists prevents the H397-F422 interaction and places helix 12 in a position in which the distance between residues K246 and E420 deviates from the optimized value of 19Å. This decreases the affinity for coactivators or even makes the interaction impossible. It has been proposed that antagonism by the 26,23-lactone 1,25-$(OH)_2D_3$ analog (TEI-9647) or by compounds ZK159222 and ZK168281, which contain an extended side chain, results from disturbing the helix 12 position (31). This is consistent with our results: ZK168492, ZK191732, and ZK168289 have an extended side chain as does the ZK159222 1,25-$(OH)_2D_3$ antagonist, and none of these compounds were able to induce recruitment of p160 coactivators or DRIP205 either in solution or bound to RXR on DNA. Thus, these VDR ligands could act as antagonists, even though some agonistic activity could be observed at high concentrations. Although ZK136607 has a chemical structure very similar to that of the natural ligand, it has little potency to induce coactivator recruitment and to transactivate. This compound binds VDR with a 10-fold lower affinity than the natural hormone, and a higher concentration appears to be necessary to achieve an agonistic behavior. In fact,

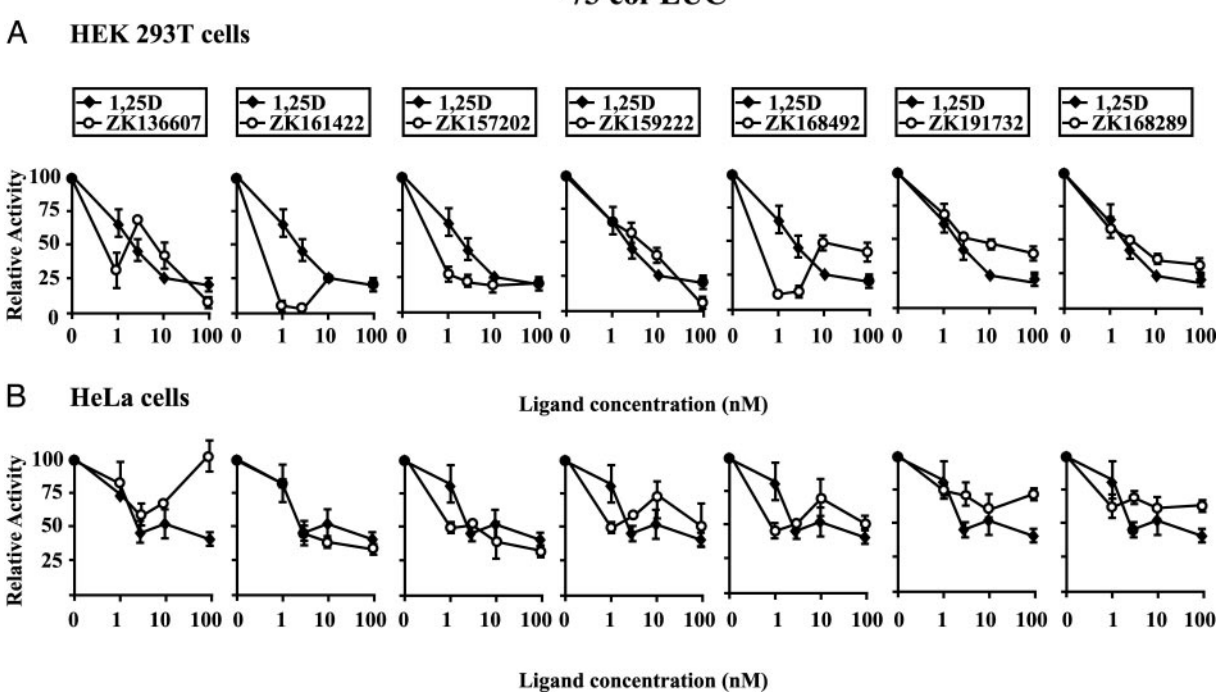


Fig. 6. Influence of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ Analogs on TPA-Dependent Transactivation of the Collagenase Promoter

A, HEK 293T cells were cotransfected with 200 ng of the −73Col-Luc reporter plasmid and 12.5 ng of VDR and RXR. B, HeLa cells transfected with 500 ng of the reporter plasmid and 80 ng of VDR and RXR. After transfection the cells were treated for 48 h with 100 nM TPA alone or in combination with the indicated concentrations of 1,25-$(OH)_2D_3$ (1,25D) or ZK analogs. Data are shown as a percentage of the luciferase activity obtained in cells incubated with TPA alone.

this compound used at 100 nM was unable to promote binding of coactivators to VDR, but at 1 μM induced partial coactivators recruitment by VDR-RXR in the supershift assays (Fig. 2C).

Prevention of VDR-RXR complex formation on DNA could be a mechanism of antagonism in 1,25-$(OH)_2D_3$ signaling. However, strong binding to the VDRE in the presence of the different compounds was found (Fig. 2C), and we have results demonstrating that both 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs equally increase binding of the VDR/RXR heterodimer to DNA (data not shown). Therefore, prevention of complex formation is not the cause for the low transcriptional activity of some ligands. In contrast, the defective interaction with coactivators appears to be the main reason why ZK168492, ZK191732, and ZK168289 have a low potency in comparison with 1,25-$(OH)_2D_3$ to transactivate VDRE reporter genes. In contrast, ZK157202, which induced coactivator recruitment, was even more potent than 1,25-$(OH)_2D_3$ in activating either a heterologous reporter or a natural promoter and could be described as a superagonist. This compound is not metabolized by the C-24 oxidation pathway, which allows it to be retained longer inside target cells, showing a higher biological activity as compared with 1,25-$(OH)_2D_3$ or ZK161422 (23).

Interestingly, some of the VDR ligands used in this study act in a cell specific-dependent manner because they lack activity in HeLa cells while presenting some agonistic activity in HEK 293T cells, or *vice versa*. Furthermore, their ability to antagonize the response to 1,25-$(OH)_2D_3$ is also quantitatively different depending on the cell context. This would describe these compounds as VDRMs. It has been suggested that ligand-selective cofactor recruitment may underlie the novel pharmacological properties of ligands that show preference for osteoblasts over intestinal cells (18). Very recently, tissue-selective nonsecosteroidal VDRMs that function as potent agonists in keratinocytes, osteoblasts, and peripheral blood mononuclear cells, but show poor activity in intestinal cells, have been described (19). Different ligands could induce different conformational changes in the receptor leading to selective coactivator recruitment. For instance, the nonhypercalcemic nonsecosteroidal analogs show differential recruitment of the coactivator, peroxisomal proliferator-activated receptor coactivator 1, to E420A mutant VDR (19). Furthermore, the secosteroidal analog 22-*oxa*-1α,25-dihydroxyvitamin D_3 has been described to induce interaction of VDR with TIF2 but not with SRC-1 or amplified in breast cancer 1 (34). This is not the case with the compounds tested here, which showed a similar profile for the recruitment of different p160 coactivators and DRIP205 (Fig. 2), although the possibility that they could recruit other coactivators selectively cannot be dismissed.

A most important finding in this work was that the ZK136607, ZK159222, ZK168492, and ZK191732

ligands have a dissociated effect, *i.e*. they have low capacity to transactivate, but they have potency to transrepress. Interestingly, these compounds that were able to inhibit activation of the RARβ2 promoter by RA have very little calcemic activity. The RARβ2 promoter contains two RA response elements (RAREs), but only the proximal βRARE appears to be sufficient to confer 1,25-$(OH)_2D_3$-mediated repression (9). We have previously shown that VDR/RXR can bind to the βRARE with high affinity, but without a defined polarity (10). Binding of the heterodimer to this element is transcriptionally unproductive for activating the promoter in response to 1,25-$(OH)_2D_3$, and the competition between active RAR/RXR and inactive VDR/RXR for DNA binding could contribute to transrepression. Accordingly, the VDR ligands tested here could generate a more stable structure of VDR/RXR on the βRARE and reduce RA-dependent transactivation.

The VDR ligands analyzed also behave as VDRMs in transrepression because their potency appears to depend on the cell context. Whereas in HeLa and HEK 293T cells all compounds were able to repress the RA response (Fig. 5), cell-specific differences have been also observed. Thus, ZK136607 was more active in HeLa than in HEK 293T cells, being even more potent than 1,25-$(OH)_2D_3$ in blocking the RA response. Thus, in addition to DNA binding competition, other mechanism/s must contribute to the inhibition, among which cell-specific differences in the metabolism of the compounds could play a role. The finding, that the VDR AF-2 domain seems to be required for the dominant negative activity of VDR (9), suggests that titration of coactivators may also be involved in the inhibition of the RA response by VDR ligands. However, all the ZK compounds, even those with an antagonistic profile that do not induce coactivators recruitment, were able to transrepress the RA response. This finding suggests that other still unidentified cofactors that bind to both RAR and VDR could be involved in the transrepression by 1,25-$(OH)_2D_3$ in HeLa and HEK 293T cells. Furthermore, these unidentified cofactors, as opposed to the classical coactivators, do not appear to require an intact AF-2 surface to mediate transrepression. In agreement with this idea, it has been shown recently that β-catenin interacts with and activates VDR in a ligand-dependent manner, under conditions in which other coactivators do not. 1,25-$(OH)_2D_3$ induces interaction between β-catenin and the AF-2 VDR point mutant E420Q (35), which has very diminished capacity to bind classical coactivators (34). Moreover, the partial antagonist ZK 159222 was also able to induce β-catenin recruitment by VDR (35).

The noncalcemic VDR ligands examined were also able to repress the response of the AP-1-containing collagenase promoter to TPA. Again, there is not a clear correlation between their agonistic activity on a VDRE and their capacity for AP-1 transrepression, demonstrating that molecular determinants governing the transrepressive activity of VDR are likely to be distinct from those ruling its transactivation potential. Furthermore, also in this case the inhibitory effect of some of the ZK compounds was more marked in HEK 293T than in HeLa cells, showing that they can function as cell-specific VDR modulators. Because the AP-1 complex regulates the expression of several genes involved in oncogenic transformation and cellular proliferation, there is considerable interest in the identification of compounds able to down-regulate AP-1 activity and thereby oppose unregulated cell growth. A number of ligands for nuclear receptors display such AP-1-repressive activity, which seems to be the basis for their beneficial therapeutic effects. That the VDR ligands analyzed here are unable to stimulate transcription efficiently but have anti-AP-1 activity is a novel finding for 1,25-$(OH)_2D_3$ analogs, but has been already described for dissociated glucocorticocoids (37) and retinoids (38–42), which can inhibit AP-1-dependent transcription, while only weakly activating GRE- or RARE-based reporter genes. These compounds could have an added therapeutic interest because they could be devoid of the deleterious side effects secondary to activation of genes containing hormone response elements.

In summary, we report here the characterization of VDR modulators that have not only cell-selective effects, but also have dissociated activity that distinguishes between transactivation and transrepression. Such compounds may be a valuable tool for studying molecular mechanisms of VDR signaling and, due to their low calcemic activity, they could be promising therapeutic agents.

MATERIALS AND METHODS

VDR Analogs

The chemical names for the 1,25-$(OH)_2D_3$ derivatives used were the following: ZK136607, (5Z,7E)-(3S)-9,10-seco-5,7,10(19)-cholestatriene-3,25-diol; ZK 157202, (5Z,7E,23E)-(1S,3R)-20-methyl-9,10-secocholesta-5,7,10(19),23-tetraene-1,3,25-triol; ZK159222, (5Z,7E,22E)-(1S,3R,24R)-1,3,24-trihydroxy-26,27-cyclo-9,10-secocholesta 5,7,10(19),22-tetraene-25-carboxylic acid butyl ester; ZK161422, (5Z,7E)-(1S,3R)-20-methyl-9,10-secocholesta-5,7,10(19)-triene-1,3,25-triol; ZK168289, (5Z,7E,22E)-(1S,3R,24R)-25-(1-oxohexyl)-26,27-cyclo-9,10-secocholesta-5,7,10(19),22-tetraene-1,3,24-triol; ZK168492, (5Z,7E,22E)-(1S,3R,24R)-1,3,24-trihydroxy-26,27-cyclo-9,10-secocholesta-5,7,10(19),22-tetraene-25-carboxylic acid 1,1-dimethyl ester; and ZK191732, (5Z,7E,22E)-(1S,3R,24R)-25-(1-methylthiazole-2-yl)-26,27-cyclo-9,10-secocholesta- 5,7,10(19),22-tetraene-1,3,24-triol.

VDR Binding Assay

The affinity of the 1,25-$(OH)_2D_3$ analogs was determined in a competition ligand-binding assay as previously described (43). Briefly, a VDR preparation was incubated with [methyl-^{3}H]1α25-vitamin D_3 (1,25-$(OH)_2D_3$) in the presence of increasing concentrations of unlabeled 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs. Bound and unbound material was

separated by absorption of the free ligand to dextran-coated charcoal. The radioactivity remaining was counted, and binding data are expressed as the IC_{50} [*i.e.* the concentration of cold ligand required to inhibit 50% of labeled 1,25-$(OH)_2D_3$ binding].

Calcemic Activity

For measurement of total calcium levels, female mice 8–12 wk old were treated with compounds by sc injection for 5 d, and urine and serum were collected 24 h after compound administration. The vehicle-treated group served as the control. For measurement of total calcium, samples were diluted 1:2 with H_2O and analyzed for calcium (millimoles/liter) by flame photometer AFM 5051 (Eppendorf, Hamburg, Germany) against a standard curve prepared from a standard solution containing 5 mM calcium (Eppendorf) as previously described (43). Calcemic activity of the different VDR ligands was expressed relative to that of 1,25-$(OH)_2D_3$. Urine calcium levels in control animals were significantly elevated (from 2 to 6 mmol/liter) after treatment with 0.03 μg/kg/d of 1,25-$(OH)_2D_3$. Significant increases of serum calcium after 1,25-$(OH)_2D_3$ administration (from 2.7 to 3.0 mmol/liter) were found with 0.1 μg/kg/d (43). The maximal concentration of 1,25-$(OH)_2D_3$ and ZK analogs used was 10 μg/kg/d.

Expression Vectors and Transfections

Expression vectors for wild-type and mutant human RXRα, VDR, and RARα are cloned in pSG5 (9). The constructs GST-ACTR, GST-TIF-2, GST-SRC-1, and GST-DRIP205 code for the nuclear receptor-interacting domains of these proteins. These plasmids have already been described (36). The −3000 PRLCAT plasmid containing the 5′-flanking region of the rat prolactin promoter fused to choramphenicol acetyl transferase (CAT) was also previously described (25, 26). This reporter (5 μg) was transfected in HeLa cells by calcium phosphate in p60 dishes. The cells were cotransfected with 2.5 μg of VDR and 0.4 μg of the GHF-1/Pit-1 transcription factor. In 4×VDRE TKLuc construct the luciferase reporter gene is driven by four copies of DR3-type VDRE from the rat ANF gene promoter (20). The R140-Luc construct contains the fragment −124 to +14 of the human RARβ2 promoter, and the construct −73Col-Luc contains the collagenase promoter fused to luciferase. These plasmids were also cotransfected with VDR and RXR as is described for 4×VDRE TK-Luc. HEK 293T cells, grown in 24-well plates, were transfected with 40 ng of 4×VDRE TK-Luc, 200 ng of R140-Luc, or 200 ng of −73Col-Luc, and the expression vectors for VDR (12.5 ng), RXRα (12.5 ng), or RARα (2.5 ng) as indicated in the figure legends. HeLa cells, also grown in 24-well plates, were transfected with 500 ng of 4×VDRE TK-Luc, R140-Luc or −73Col-Luc and VDR (80 ng), RXRα (80 ng), and RARα (16 ng), as indicated in the figures. In all cases, after transfection cells were plated in medium containing hormone-stripped serum and, after an overnight incubation, treatments were started, and luciferase or CAT activity was determined after 48 h. Experiments were performed with triplicate cultures, and each experiment was repeated at least three times. Data are represented as means ± SDS.

Limited Proteolytic Digestion

Limited proteolytic assays were performed as described (36). *In vitro*-translated [^{35}S]VDR was incubated in the presence of ethanol or 10 μM 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs for 20 min at room temperature. The receptors were then incubated for 2 min with 10 or 30 μg/ml of trypsin. Proteolysis was stopped by adding SDS sample buffer, and the proteolytic fragments were separated by SDS-PAGE in a 12% polyacrylamide gel and identified by autoradiography.

Gel Retardation Assays

Mobility shift assays were performed with 2.5 μl of *in vitro*-translated VDR and RXR in the presence and absence of 400–600 ng of recombinant GST-fused SRC1, ACTR, TIF 2, or DRIP205 and the consensus DR3 oligonucleotide 5′-AGCTCAGGTCAAGGAGGTCAG-3′ as previously described (25). 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs were used at 1 μM.

GST Pull-Down Assays

Protein-protein interactions were performed with 5 μl of *in vitro* translated [^{35}S]VDR and the fusion proteins of GST-ACTR, GST-TIF-2, GST-SRC-1, or GST-DRIP205, or GST as a control (25, 26). Fifteen minutes before and during the binding reaction, [^{35}S]VDR is incubated in the presence of 100 nM 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs. The bound proteins were analyzed by SDS-PAGE in a 10% polyacrylamide gel and identified by autoradiography.

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Discusión

Discusión

1. Papel activo del RXR y su ligando el ácido 9-*cis*-retinoico en los heterodímeros TR/RXR y VDR/RXR

Como describíamos al comienzo de esta tesis, la mayoría de receptores no esteroideos ejercen sus acciones sobre la transcripción en forma de heterodímeros con el RXR. Al contrario que la homodimerización, la heterodimerización permite, en principio, una fina regulación de la acción de los NRs por medio de la combinación de ligandos, así como la regulación de repertorios alternativos de genes diana, lo cual es especialmente interesante desde un punto de vista farmacológico. Se han definido 3 tipos de heterodímeros según el papel más o menos activo del RXR dentro de la pareja: permisivos, no permisivos y condicionales (179). Tradicionalmente se han considerado como "no permisivos" para la acción del ligando del RXR a los heterodímeros RAR/RXR, TR/RXR y VDR/RXR, en los que la actividad transcripcional del RXR se encontraría suprimida debido a que la propia heterodimerización evitaría la unión del 9-*cis*RA a su receptor (62). A pesar de describirse que en el contexto de estos heterodímeros RXR aún retiene la capacidad de unir ligando (192), y de sugerirse la importancia de la modificación alostérica ejercida por RXR sobre VDR (17), el papel del RXR en estos heterodímeros comenzó a ser realmente revisado cuando se describió que este receptor era capaz de reclutar coactivadores y estimular la transcripción en respuesta a sus propios agonistas en el contexto del heterodímero RAR/RXR (66). Además de esta importante evidencia molecular, encontramos en la literatura múltiples casos que apuntan hacia un claro papel de los retinoides en la señalización mediada por TR (76, 108, 119, 165, 176, 211) y VDR (21, 89, 95, 160, 187). Gran parte de nuestro trabajo ha estado encaminado hacia la demostración de que en cada uno de estos heterodímeros existe una importante función del RXR, más allá de ser un mero "compañero silencioso" que contribuye a incrementar la afinidad del heterodímero por el DNA.

Utilizando como modelo el gen de la prolactina de rata, identificamos la existencia de un TRE en su promotor que se regulaba a través de la acción de un heterodímero permisivo TR/RXR. Este heterodímero, al igual que RAR/RXR, es capaz de unir coactivadores y activar la transcripción en respuesta a cada uno de los ligandos de la pareja heterodimérica independientemente, siendo sinérgicamente activado cuando ambos agonistas se proporcionan conjuntamente. Más aún, apuntamos que este fenómeno no es una particularidad del gen de prolactina de rata, sino que se trata de una característica intrínseca del heterodímero, ya que el TRE de prolactina es capaz de conferir capacidad de respuesta tanto a T3 como a 9-*cis*RA a un promotor heterólogo y por otro lado, utilizando elementos de respuesta consenso conseguimos idénticos resultados. El uso de retinoides específicos y de la línea celular 235-I que expresa bajos niveles de TR demuestran que la activación transcripcional por T3 y 9-*cis*RA se produce a través del heterodímero TR/RXR.

Por su parte, el heterodímero VDR/RXR se comporta de una manera similar. También es capaz de reclutar coactivadores y de estimular la transcripción de diversas construcciones reporteras y de un

gen natural diana de la VitD, *cyp*24, en respuesta al agonista de cada componente del heterodímero. Se observa además un claro efecto cooperativo, en algunos casos sinérgico, asociado a la presencia de los dos ligandos de la pareja. La inhibición de la expresión de VDR mediante técnicas de siRNA y el uso de retinoides específicos demuestran que estos efectos están producidos a través del heterodímero VDR/RXR. Por tanto, nos encontramos ante otro caso de heterodímero, previamente descrito como no permisivo para la acción del RXR, en el que este receptor no actúa como pareja "silenciosa".

Cobra especial importancia el papel del ligando natural del RXR, al ácido 9-*cis*RA, en la señalización por TR o VDR en el caso de que existan defectos en alguno de los componentes implicados en la correcta función del heterodímero. Así, el uso de receptores mutados en un residuo de ácido glutámico del dominio AF-2, indispensable para la activación transcripcional dependiente de ligando, demuestra claramente tanto en experimentos de unión de coactivadores, como en ensayos de transactivación, que el efecto de la mutación de residuos críticos para la actividad de TR o VDR puede ser revertido significativamente cuando el ligando de estos receptores se combina con el agonista de RXR. El hecho de que se hayan encontrado mutaciones puntuales de este tipo en la región AF-2 de la isoforma β de TR en pacientes con síndrome de resistencia a hormonas tiroideas (43), y en el VDR en pacientes con raquitismo hereditario sin alopecia con resistencia a VitD (126), permite sugerir un posible uso terapéutico de los rexinoides en este tipo de dolencias causadas por un defecto en la transactivación del receptor emparejado con RXR. Por otro lado, hemos de destacar el papel de los propios rexinoides endógenos en este tipo de fenómenos, ya que la mutación del residuo E420 en el VDR produce un fenotipo suavizado donde el raquitismo cursa sin alopecia, lo que podría deberse a una activación transcripcional residual ocasionada por niveles suficientemente efectivos de rexinoides en ciertos tejidos.

Otro caso de recuperación de la respuesta transcripcional por el ácido 9-*cis*RA en estos heterodímeros lo encontramos cuando utilizamos coactivadores mutados en los motivos de interacción con los NRs. En especial, la mutación del segundo motivo LxxLL bloquea la unión de los coactivadores a los heterodímeros correspondientes en respuesta a T3 o VitD. Sin embargo, la ocupación del RXR por su ligando es capaz de proporcionar al heterodímero una estructura competente para la unión del coactivador aunque su motivo de interacción principal se encuentre mutado. Puesto que se ha comprobado la unión de una sola molécula de coactivador por cada heterodímero (58, 208), muy probablemente la presencia de los dos agonistas promovería el reclutamiento del coactivador a través de las cajas LxxLL I y III, uniendo cada miembro de la pareja uno de estos motivos. De hecho se ha propuesto que el sinergismo observado cuando los dos ligandos se encuentran presentes se originaría de la unión cooperativa de dos motivos de interacción del coactivador con un único heterodímero (66). Además, como muestran nuestros ensayos de retardo en gel realizados con los heterodímeros TR/RXR y VDR/RXR, el reclutamiento de coactivadores por cada uno de los ligandos de la pareja viene representado por una banda de superretardo de distinta movilidad, lo que podría significar que cada uno de los miembros del heterodímero estaría utilizando una superficie diferente de interacción con los coactivadores. Aparentemente no existiría una preferencia en los miembros del heterodímero por el motivo a unir dado que la mutación de estas cajas tiene efectos tanto en el reclutamiento mediado por 9-

*cis*RA como por los ligandos de sus parejas. Podría ocurrir también que el motivo que uniese cada receptor viniese definido por la estructura más o menos estable que desarrollasen estos heterodímeros sobre los distintos elementos de respuesta en diferentes ambientes celulares.

Un último caso de situación transcripcional adversa en la que se pueden encontrar estos heterodímeros es la presencia de un ligando antagonista o con escasa capacidad transactivadora. En particular, el análogo de la VitD ZK159222, de escaso potencial agonista, nos vuelve a proporcionar un claro ejemplo de la importancia del 9-*cis*RA en la señalización mediada por estos heterodímeros. Este ligando por si solo no es capaz de producir reclutamiento de coactivadores, ni de transactivar de manera efectiva. Sin embargo, la combinación con el 9-*cis*RA produce un reclutamiento de coactivadores y una transactivación casi equiparable a la resultante de la combinación del rexinoide con la VitD. Nuevamente la unión del ligando del RXR permite la consecución de una estructura heterodimérica agonista aunque su compañero se halle unido a un ligando inactivo. ZK159222 posee una cadena lateral extendida que no permite un correcto posicionamiento de la H12 en el VDR, lo que le impediría reclutar coactivadores eficazmente (194). La unión de ligando al RXR podría producir un cambio conformacional en su pareja reposicionando la hélice de activación hacia la posición agonista.

En el caso del heterodímero TR/RXR sería interesante estudiar como se comportan los distintos ligandos agonistas y antagonistas de TR recientemente desarrollados (205), tanto en el reclutamiento de coactivadores como en transactivación por si solos y en presencia de 9-*cis*RA, pues es probable que posibles antagonistas tornasen hacia papeles agonistas como en el caso del análogo de la VitD ZK159222.

Aunque cada receptor del heterodímero puede promover el reclutamiento de coactivadores de forma autónoma, parece existir un importante "cross talk" entre los dos miembros de la pareja, que no funciona del mismo modo en los dos tipos de heterodímeros estudiados. Por ejemplo, la deleción del dominio AF-2 del TR impide el reclutamiento de coactivadores no solamente en respuesta a T3, sino también a 9-*cis*RA, y esto se traduce en una pérdida de respuesta transcripcional a los dos compuestos en células que expresan este receptor truncado. Recuerda esto al efecto del "ligando fantasma", observado con otros heterodímeros como LXR/RXR, en los cuales la unión del ligando del RXR permite la activación del LXR (209), o bien la unión de un ligando sintético del RXR que mimetiza los efectos observados cuando RAR está unido a su ligando (172). Sin embargo, en el caso de VDR/RXR, la deleción del dominio AF-2 del VDR no tiene efectos sobre el reclutamiento de coactivadores en respuesta a 9-*cis*RA, al igual que la deleción de dicho dominio en el RXR no tiene efectos sobre la unión de coactivadores mediada por VDR, o por TR. La particularidad del heterodímero VDR/RXR reside en que la mutación puntual de residuos de la H12 de RXR tiene efectos más drásticos que la deleción en si, ya que no solamente bloquea la unión de coactivadores en respuesta a 9-*cis*RA, sino que disminuye en gran medida la producida en respuesta a la vitamina. De nuevo las respuestas desencadenadas en un receptor repercuten en el funcionamiento de su compañero.

Utilizando el análisis estadístico denominado SCA (análisis de acoplamiento estadístico) que permite detectar la co-evolución de residuos aminoacídicos funcionalmente relacionados (121) se han

identificado una serie de residuos críticos para la función alostérica cuya mutación puede convertir un heterodímero permisivo en uno condicional, o también consentir la unión de ligandos alternativos para un receptor dado (179). Así la mutación de estos residuos en el LXR hace que la respuesta al ligando del RXR sólo se produzca en presencia del ligando de su pareja. Mutaciones equivalentes en el RXR no presentan apenas efectos, indicando una dominancia funcional dentro del heterodímero de las parejas de RXR. En el caso de VDR/RXR esta dominancia no sería tal, puesto que la mutación de residuos del dominio AF-2 del RXR, aunque no se identifiquen como parte de esta red alostérica, tiene efectos en la respuesta al ligando de su pareja, lo que proporciona una interesante particularidad a este heterodímero. Estos resultados muestran una precisa comunicación intermolecular entre los miembros de la pareja donde los LBDs de ambos receptores se erigen como complejos dominios de señalización alostérica capaces de integrar los múltiples tipos de interacciones moleculares que modulan la activación transcripcional. Fenómenos como el del "ligando fantasma" indican que el tipo de regulación alostérica de cada pareja de receptores tendría la clave que establecería el grado de permisividad de los heterodímeros del RXR.

Las parejas del RXR incluyen receptores que reconocen ligandos metabólicos con baja afinidad como PPAR, LXR y FXR y receptores endocrinos que reconocen sus ligandos con alta afinidad como TR y VDR (34). Se ha relacionado la permisividad de los heterodímeros del RXR con receptores para ligandos metabólicos, comportándose como no permisivos los receptores endocrinos. El heterodímero condicional RAR/RXR representaría un intermedio evolutivo ya que reconocería lípidos derivados de la dieta (vitamina A) como los receptores permisivos, aunque regularía la morfogénesis y el desarrollo al modo de los receptores endocrinos. En el caso de TR/RXR y VDR/RXR, nuestros datos indican una clara condicionalidad para la acción del ligando del RXR. Los heterodímeros condicionales, según los definen estos autores, presentarían escasa respuesta al agonista del RXR y una activación sinérgica al combinarse con el ligando de su pareja, mientras que los permisivos presentarían una respuesta al ligando de RXR de magnitud semejante a la del ligando de su pareja y más bien un efecto aditivo en la combinación de ligandos. Aunque en la mayoría de los contextos analizados TR/RXR presenta efectos sinérgicos y VDR/RXR más bien efectos aditivos en la combinación de ligandos, en relación a la respuesta al ligando del RXR, que es lo que realmente define la permisividad de los distintos heterodímeros, ambos heterodímeros presentan una respuesta a 9-*cis*RA sustancialmente menor que la producida por la VitD o la T3, por lo que se definiríamos a estos heterodímeros como condicionales.

Nuestros resultados demuestran que la permisividad o condicionalidad de estos heterodímeros viene influida en gran medida por el contexto celular, especialmente por la razón entre los niveles de coactivadores y correpresores. En el caso de TR/RXR, la respuesta al 9-*cis*RA varía entre tipos celulares, produciéndose en Hela y GH4C1 pero no en CV-1. Sin embargo, en este último tipo celular la sobre-expresión de coactivadores permite la estimulación del promotor de prolactina por el 9-*cis*RA. Esto pone de manifiesto la importancia de los niveles de coactivadores/correpresores presentes en la célula para las respuestas transcripcionales producidas por uno u otro ligando de la pareja, así como por la combinación de ambos. Se encontraría, por tanto, mayor facilidad para la acción del 9-*cis*RA en aquellos ambientes

celulares con un alto contenido en coactivadores. El que se encuentren efectos aditivos o sinérgicos dependiendo de la línea celular analizada posiblemente esté supeditado a una cantidad adecuada y balanceada de ambos tipos de correguladores. Así, las diferencias de permisividad entre unos y otros heterodímeros podrían venir además influidas por el reclutamiento preferencial de determinados cofactores según el tipo de heterodímero y de la ocupación de éste por los distintos ligandos. Cabe también destacar que en determinados contextos el 9-*cis*RA produce mayores respuestas transcripcionales a través de TR/RXR que las producidas por la T3. Esto podría deberse además de al tipo de correguladores presentes y su abundancia relativa, al metabolismo particular que puedan sufrir los ligandos dentro de la célula. Se abre entonces un enorme abanico de posibilidades de actuación de los heterodímeros del RXR dependiendo del tipo de heterodímero y del contexto celular en el que se encuentren.

Se ha identificado un ácido biliar secundario como ligando endógeno de VDR, el ácido litocólico (LCA) (124), lo que relacionaría al VDR con los receptores metabólicos, puesto que la afinidad por este compuesto es bastante menor que la afinidad por su ligando clásico, la VitD. Por otro lado, la mutación en residuos integrantes de la red alostérica del VDR no afecta a la respuesta a VitD y sí a LCA (179), que sería el ligando metabólico relacionado con la permisividad. Desde un punto de vista evolutivo se propone que los receptores endocrinos provendrían de un antecesor metabólico y habrían ido ganando la capacidad de unir ligandos de alta afinidad para regular respuestas fisiológicas complejas como el desarrollo, la reproducción y el mantenimiento de la homeostasis global. Ya que este tipo de respuestas complejas necesitarían una regulación mucho más precisa que las mediadas por sensores metabólicos, se habría tendido a una independencia de la señalización mediada por rexinoides. La capacidad de estos heterodímeros "endocrinos" de responder a través del RXR podría representar un mecanismo de emergencia o de compensación por el que en determinados ambientes celulares con un conjunto específico de cofactores o donde predominen determinados ligandos, las respuestas transcripcionales mediadas a través del RXR cobrarían especial importancia.

Desde un punto de vista estructural, el análisis cristalográfico del heterodímero permisivo PPAR/RXR muestra cómo, a diferencia de otros heterodímeros, la interacción entre el extremo C-terminal de PPAR y la H10 del RXR puede estabilizar la H12 de PPAR en la conformación agonista incluso en ausencia de ligando (65). Este receptor posee una cavidad de unión a ligando amplia y un giro entre las hélices H2' y H3 particularmente flexible que le permite modos alternativos de entrada de ligando (139, 216). Curiosamente, el VDR también posee un giro entre las hélices 1 y 3, muy flexible y largo, lo que hace necesaria su deleción para lograr la cristalización del LBD (163). Esta estructura desorganizada podría ser la que permitiese al VDR unir LCA conservando así la capacidad vestigial de unir ligandos metabólicos propia de los heterodímeros permisivos clásicos. Más aún, PXR, que comparte un 44% de su secuencia con VDR (2) y es capaz de unir entre otros ligandos LCA (186, 215), aunque con menor afinidad que VDR (124) también posee este tipo de giro flexible entre H1 y H3 (204). Estas observaciones sugieren una base estructural para la permisividad o condicionalidad en los heterodímeros

de RXR. Sin embargo, quedarían por definir aún las características estructurales que definen a los heterodímeros condicionales TR/RXR y RAR/RXR.

Una importante observación de este trabajo es el papel del ligando del RXR en la regulación de una respuesta fisiológica compleja como es la diferenciación en las células de cáncer de colon SW480ADH (144). El 9-*cis*RA, al ser combinado con bajas dosis de VitD o con el agonista parcial ZK159222, produce la diferenciación de estas células hacia un fenotipo epitelial que se refleja en un aumento de la expresión de E-Cadherina. A su vez se ha descrito que el LCA podría jugar un importante papel en la formación de cáncer de colon (77, 140). Tanto la VitD como el LCA y sus derivados estimulan la expresión del gen detoxificador de LCA, *cyp*3A (124, 193), estableciéndose el VDR como un importante sensor de estos ácidos biliares secundarios característicos de las dietas ricas en grasas que se han asociado con alto riesgo de cáncer de colon (134). Sería probable que en estos casos el 9-*cis*RA también fuese capaz de cooperar con la Vit D y estos ácidos biliares en la estimulación del heterodímero VDR/RXR desencadenando los mecanismos consiguientes de protección antitumoral. Ya que la afinidad de estos ácidos biliares por el VDR es menor que la de la VitD, la combinación con el 9-*cis*RA podría incrementar la sensibilidad del sistema protector en estados de bajos niveles de VitD; además esta situación no produciría la hipercalcemia derivada de los tratamientos con dosis farmacológicas de VitD. Así pues, para paliar los efectos de las dietas altas en grasas podrían ser necesarios aportes adecuados tanto de vitamina A como de vitamina D.

En conjunto nuestros datos parecen indicar que la heterodimerización con RXR de los NRs de la subfamilia II tiene un claro papel en la señalización mediada por estos receptores y muestran la necesidad de revisar la clasificación de estos heterodímeros en 3 subtipos. Según nuestros datos, existirían solamente 2 tipos de heterodímeros, permisivos o condicionales; aunque quizás la definición más sencilla sería un único tipo de heterodímeros con distintos grados de permisividad según la magnitud e importancia de las respuestas transcripcionales mediadas a través del RXR.

2. Unión de correpresores al heterodímero VDR/RXR en respuesta al agonista de VDR

Los receptores nucleares regulan la transcripción génica a través del reclutamiento de correguladores: coactivadores y correpresores. El reclutamiento de coactivadores conduce a una descompactación de la cromatina a través de actividades acetilasas y metilasas de histonas que conlleva una activación transcripcional, mientras que los complejos correpresores incluyen actividades desacetilasas de histonas que producen una compactación de la cromatina y un estado de represión transcripcional.

El modo de unión de los complejos correpresores difiere entre los distintos NRs. Los receptores no esteroideos como RAR y TR, en ausencia de ligando actuarían como potentes silenciadores transcripcionales uniendo correpresores como SMRT o NCoR, que se liberarían tras la unión de un agonista (37, 70, 86). Los receptores esteroideos como ER, no unen correpresores en ausencia de la hormona, por lo que no se detecta una represión de la transcripción por los receptores vacíos. La

interacción con un ligando agonista produciría el reclutamiento de coactivadores y la consiguiente activación transcripcional. Sin embargo, la unión de antagonistas produce un desplazamiento de la H12 con respecto a la posición agonista permitiendo la unión de correpresores al receptor y el silenciamiento génico (24, 178, 181, 228).

El receptor de la VitD aunque pertenece a la misma subfamilia que TR y RAR y comparte con ellos múltiples características, no produce una clara represión transcripcional en ausencia de ligando, por lo que su capacidad como silenciador transcripcional siempre ha sido objeto de controversia. A lo largo de este trabajo hemos demostrado que el heterodímero VDR/RXR posee capacidad de reclutamiento de correpresores como SMRT y NCoR, con la particularidad de hacerlo en respuesta a agonistas del VDR. Además, esta interacción se incrementa fuertemente cuando la H12 del RXR es delecionada, causando una disminución de la activación transcripcional en respuesta a la vitamina. En la literatura encontramos varios estudios tempranos que muestran el efecto represor de la deleción de dicha región del RXR. Ensayos de represión con construcciones del RXR unido al DBD de Gal4 muestran que la deleción de H12 convierte a RXR en un potente silenciador transcripcional (113, 226). Asimismo, la deleción de esta hélice causa una clara disminución de la transactivación mediada por sus heterodímeros con RAR, VDR y TR (171).

Los ensayos de retardo en gel, así como los ensayos de un híbrido en células 293-T muestran un claro reclutamiento de correpresores al VDR/RXR en presencia de VitD, que además es estrictamente dependiente de la capacidad agonística del ligando de VDR utilizado. Lo que es aún más importante, los ensayos de inmunoprecipitación de cromatina (ChIP) confirman el reclutamiento de SMRT y NCoR en respuesta a VitD al promotor de *cyp*24, un gen diana para los heterodímeros VDR/RXR. El reclutamiento concomitante de HDAC3, un componente fundamental de los complejos de estos correpresores (74, 80, 114), refuerza la evidencia de esta unión. Además este reclutamiento se produce de una forma cíclica, lo que podría ser consecuencia de los ciclos de unión de VDR al promotor de *cyp*24 observados recientemente (199). Esto ocurre también en genes diana del receptor de estrógenos (ER) para el reclutamiento de distintos correguladores en respuesta al ligando (129, 130, 159). Por tanto, nuestros datos demuestran que el heterodímero VDR/RXR es capaz de reclutar *in vivo* correpresores en respuesta a VitD. La disminución de la respuesta transcripcional a la VitD causada por la sobre-expresión del mutante de RXR carente de H12 se debería al aumento del reclutamiento de correpresores. Esto indica la influencia de este reclutamiento en la respuesta transcripcional a la VitD.

Aunque inicialmente se describió la ausencia de interacción entre los correpresores y VDR (86), otros estudios posteriores indicaban la interacción del VDR con correpresores y su posterior liberación tras la unión del ligando (85, 188). Nuestros datos muestran una interacción en solución de VDR con SMRT que la VitD no es capaz de liberar. Además, en ensayos de "doble híbrido" no detectamos interacción de VDR con correpresores en ausencia de ligando a pesar de la existencia de trabajos que describen dicha interacción en este tipo de experimentos (188, 213). Sin embargo, observamos una débil represión de la transcripción cuando este receptor se halla fusionado al dominio de unión al DNA Gal4. Esta ligera capacidad se pierde cuando el VDR se encuentra heterodimerizado con RXR. Así la

heterodimerización con RXR impide el silenciamiento transcripcional por el receptor vacío, pero permite la unión de correpresores en respuesta a la VitD cuando los receptores se encuentran sobre un elemento de respuesta adecuado, tal y como demuestran los ensayos de retardo en gel. Para otros receptores no esteroideos la heterodimerización interviene también en la unión de correpresores. Por ejemplo, los mutantes de TR incapaces de heterodimerizar con RXR a su vez son defectuosos en represión (9, 155) y la heterodimerización con RXR a través de una interfase de heterodimerización heteróloga restaura la represión por estos mutantes (227). En el caso de VDR, la heterodimerización, que constituye la principal forma de actuación de este receptor en la célula, evitaría el silenciamiento transcripcional por el receptor vacío pero permitiría la unión de correpresores en respuesta a la VitD. El reclutamiento de correpresores podría así limitar la intensidad de la respuesta a la vitamina.

Existe una descripción previa del reclutamiento del correpresor NCoR al promotor del gen de la 25-hidroxivitamina D3 1-α-hidroxilasa (*cyp*27B1) en respuesta a VitD (64, 132). Sin embargo, este gen contiene un VDRE negativo y se reprime por VitD. En este caso el heterodímero no se encuentra directamente posicionado sobre el VDRE, sino que lo hace a través de un factor de transcripción bHLH intermediario (Murayama et al., 2004). Se podría pensar que el correpresor estaría participando en el mecanismo de transrrepresión y que su reclutamiento sería específico para genes regulados negativamente. Sin embargo, a la luz de nuestros datos estos resultados podrían ser consecuencia de la capacidad intrínseca del agonista del VDR para producir el reclutamiento de correpresores al heterodímero cuando éste se encuentra en un posicionamiento adecuado.

El heterodímero VDR/RXR es capaz de reclutar tanto SMRT como NCoR en respuesta a la VitD, aunque muestra una menor afinidad por este último. Estos resultados concuerdan con otros que describen una interacción más fuerte de VDR con SMRT (188). El caso opuesto sería el TR que uniría NCoR con mayor afinidad que SMRT. VDR/RXR podría entonces reclutar estos dos correpresores relacionados tanto estructuralmente, como funcionalmente. Se ha descrito la existencia de un correpresor, Alien, que se uniría al VDR en ausencia de ligando, para el que se ha sugerido un papel en la represión transcripcional mediada por VDR (152). Aunque estos autores indican la interacción en solución de Alien con el VDR vacío, nosotros no hemos sido capaces de detectar la unión de este correpresor con el heterodímero sobre el DNA ni en presencia, ni en ausencia de VitD (observaciones no publicadas). Puesto que Alien posee una estructura y un modo de actuación que diverge del de SMRT y NCoR, probablemente emplee para su función dominios moleculares del receptor diferentes a los implicados en el reclutamiento de los correpresores clásicos.

Quizás la observación más interesante de este estudio sea el hecho de que la unión de SMRT y NCoR a VDR/RXR se produzca en presencia de un agonista del VDR. A diferencia de otros modelos de reclutamiento de correpresores donde la interacción se produce en ausencia de ligando o bien en presencia de antagonistas, en el caso de VDR/RXR la magnitud del reclutamiento es directamente proporcional a la capacidad agonística del ligando usado. Puesto que se trata de un reclutamiento mediado por agonista, no es de extrañar que la mutación puntual o la deleción de la región AF-2 del VDR tenga efectos drásticos sobre la unión de correpresores. Defectos en este dominio del VDR, en el

cual reside la activación transcripcional dependiente de ligando y el reclutamiento de coactivadores, impiden también el reclutamiento de correpresores al heterodímero. Esto nos indicaría la necesidad de una conformación agonista en el VDR, que vendría reflejada en último término por el reposicionamiento de la H12 de éste, permitiéndose así la interacción con el correpresor. El papel de la H12 del VDR en el reclutamiento de corepresores es opuesto al que ejerce este dominio en el caso de TR y RAR, ya que su mutación o deleción en estos receptores potencia la interacción con correpresores y dificulta su liberación del heterodímero correspondiente tras unirse el ligando (37, 118).

El hecho de que mutaciones en la superficie de interacción del RXR con los correpresores, formada por residuos de las hélices 3, 4 y 5, tengan mucho mayor efecto que en las equivalentes en el VDR, indica que si no exclusivamente, si mayoritariamente, el reclutamiento de correpresores al heterodímero se produce a través del RXR. Nuestros resultados sugieren un modelo en el que la unión de un ligando agonista al VDR desencadenaría el reclutamiento de correpresores por su pareja heterodimérica, el RXR. Estas observaciones no solamente destacan la contribución fundamental del RXR en la unión de correpresores a VDR/RXR, sino que una vez más prueban la intensa comunicación alostérica que se produce en el seno de esta pareja de receptores.

A diferencia de los agonistas de VDR, el ácido 9-*cis*RA produce la liberación de los correpresores del heterodímero VDR/RXR, lo cual indica el papel antagónico de ambos ligandos de la pareja en cuanto a la unión de estos cofactores. No solamente el agonista natural del RXR produce esa liberación, sino que un agonista sintético de menor potencia transactivadora en heterodímeros que en homodímeros (109), o un antagonista (48), producen el mismo tipo de efecto. Parece ser que el único requerimiento para la liberación de los correpresores por el RXR es la ocupación de la cavidad de unión a ligando por el mismo y no la configuración agonista de este receptor. Por otro lado, el heterodímero truncado VDR/RXRΔH12, que une con mayor potencia correpresores en respuesta a VitD, es capaz de liberarlos tras la unión del ligando del RXR a pesar de carecer del dominio AF-2. Por tanto, en este juego de unión y liberación de correpresores por los ligandos del heterodímero, no solamente son cruciales los cambios de posición de la H12 de ambos receptores, sino que la unión del ligando al RXR debe provocar la reestructuración de otras regiones de la molécula que es suficiente para producir la liberación de correpresores incluso en ausencia de la H12. Se sabe que la unión del ligando provoca también el movimiento de la H11 (138) y la inclinación de la H3 (131). Este tipo de cambios podrían proporcionar una configuración que permitiría la liberación de los correpresores del heterodímero en presencia de agonistas y antagonistas a pesar de la ausencia de la H12. Parece que la principal diferencia en el comportamiento de agonistas y antagonistas radica en que provocan distintas posiciones de la H12, por tanto, no es de extrañar que en ausencia de ésta, ambos presenten un mismo comportamiento en cuanto a la liberación de correpresores.

Como hemos mencionado la H12 del VDR juega un papel crucial en el reclutamiento de correpresores al heterodímero. Su cambio de posición tras la unión de la VitD, de alguna manera produciría un cambio alostérico en el RXR que haría accesible la superficie de unión a correpresores de este receptor. Por el contrario, la H12 del TR en el contexto de la represión por TR/RXR (226) ejerce

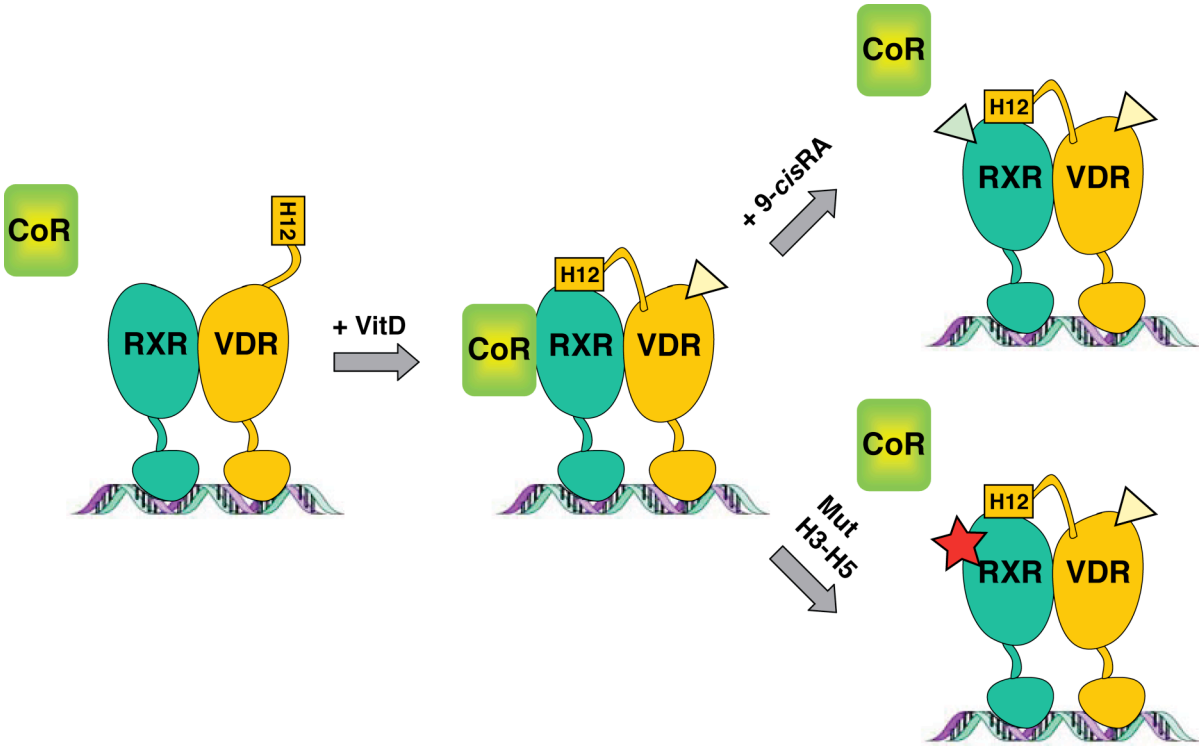
una función bien distinta. En este caso sería la encargada tras la unión de la T3 del proceso inverso, es decir, de volver a reposicionar la H12 del RXR en la conformación en la que la superficie de interacción con correpresores del RXR quedaría ocluida, liberándose entonces el correpresor. La H12 de RXR enmascararía la superficie de interacción de este receptor con los correpresores, por lo que la actividad represora de los homodímeros de RXR es prácticamente indetectable (87). La deleción de este dominio o bien la heterodimerización con otros receptores como TR, dejarían al descubierto la superficie de unión produciéndose el reclutamiento del correpresor. La heterodimerización permitiría albergar la H12 del RXR en el surco hidrofóbico de unión de coactivadores del TR de acuerdo con estudios de mutación de residuos de dicha zona así como por la gran homología que presenta la secuencia de la H12 del RXR con el motivo LxxLL de los coactivadores (226). No se trata del único caso, la H12 de RXR interacciona también con el surco de unión de coactivadores de RAR (208). Este tipo de comunicaciones intermoleculares que llevan al alojamiento de la H12 de un receptor en su pareja se produce entre otros heterodímeros (65, 218) y también en homodímeros (23, 139, 191). Se pone de manifiesto el importantísimo papel que juegan los dominios AF-2 de la pareja en el reclutamiento y liberación de los correpresores, debido a la extraordinaria capacidad de movilidad de estos dominios en el seno de estas moléculas.

VDR/RXR es pues un heterodímero particular para la unión de correpresores presentando una situación diferente con respecto a TR/RXR y PPAR/RXR. En el caso de TR/RXR, el reclutamiento se produce en ausencia del ligando y la llegada del mismo liberaría la interacción, incluso cuando se trata de un antagonista (205). Podrían no obstante existir antagonistas de TR que incrementaran la unión de correpresores, ya que para RAR se definen dos tipos de antagonistas, los que liberarían correpresores y los que estimularían su unión (66). Por otro lado, PPAR aunque es capaz de interaccionar con correpresores en solución, presenta un reducido reclutamiento cuando forma heterodímeros con RXR (224). Trabajos más recientes describen la capacidad del heterodímero PPAR/RXR vacío para la unión de correpresores en determinados contextos (105, 177, 185). La falta de interacción de PPAR con la H12 de RXR (226) sería la responsable del reducido reclutamiento de correpresores por el heterodímero PPAR/RXR en ausencia de ligando. Muy probablemente, esa falta de interacción con la H12 del RXR sea la que produzca el mismo efecto en el VDR vacío dificultando el reclutamiento de correpresores al heterodímero. Este tipo de comportamientos sin duda ha de radicar en algún rasgo estructural de estos receptores. TR y VDR presentan muy pocas diferencias estructurales en su LBD, fundamentalmente la presencia de un dominio desorganizado entre las hélices 2 y 3 del VDR. Ya que PPAR posee una hélice α adicional, la H2', VDR aparece como un intermedio entre TR y PPAR, albergando una inserción, no presente en TR, pero que en PPAR ha derivado hasta una nueva hélice α. Funcionalmente observamos el mismo gradiente, TR uniría correpresores en ausencia de ligando, VDR lo haría en presencia de agonista y finalmente PPAR, sólo podría lograr un fuerte reclutamiento de correpresores a través de la unión de antagonistas (217). A su vez se sucederían toda una serie de modificaciones en la posición de la H12 del RXR variando en función del tipo de heterodímero y ligandos implicados.

Parece claro que la VitD media la activación de sus genes diana a través del reclutamiento de coactivadores. Sin embargo, como demostramos en ensayos ChIP, los correpresores también se reclutan al promotor del gen diana *cyp*24 en respuesta a VitD. Si la unión al heterodímero de ambos tipos de correguladores es mutuamente excluyente (66) ya que comparten similares superficies de interacción (137, 150), surge la pregunta de cuál sería el tipo de corregulador que se reclutaría preferentemente tras la llegada de la vitamina. Los ensayos de competición en los que la adición de coactivadores desplaza a la de correpresores demostrando una mayor afinidad por los primeros, nos brindan la primera respuesta inmediata a esta cuestión. Por ello, las respuestas fisiológicas a la VitD mediadas por VDREs son de tipo activador. Sin embargo, la capacidad del heterodímero de unir correpresores en presencia de agonista nos hace pensar que su comportamiento en transactivación pudiera venir en gran media influido por la relación entre coactivadores y correpresores existente en cada ambiente celular. La demostración experimental de esta hipótesis se encuentra en el hecho de que la inhibición de la expresión de SMRT y NCoR por técnicas de siRNA aumente la respuesta transcripcional a la vitamina. Esto pone de manifiesto la implicación del reclutamiento de correpresores en la señalización por VitD, que limitaría la activación transcripcional mediada por los coactivadores. El hecho de que los niveles de H4 acetilada en el promotor de *cyp*24 en respuesta a la VitD aumenten de forma notoria en células en las que la expresión de SMRT y NCoR se reduce utilizando siRNAs específicos, refuerza la importancia de la competición *in vivo* entre coactivadores y corepresores en las respuestas transcripcionales mediadas por el heterodímero VDR/RXR. Además de por el balance entre coactivadores y correpresores (30, 66), las respuestas transcripcionales a los receptores nucleares podrían venir determinadas también por el contexto génico. Así se ha descrito recientemente que dependiendo del tipo de genes regulados, se podrían unir bien correpresores o coactivadores a los heterodímeros PPAR/RXR (72).

La posibilidad en principio paradójica de que se produzca reclutamiento tanto de coactivadores como de correpresores de forma dependiente de agonista, no sorprende cuando pensamos que existen otros correpresores como RIP140 o LCoR que se unen a los NRs en respuesta a ligando agonista (59, 197). Estos correpresores también competirían con los coactivadores por los receptores y pueden modular la respuesta a los ligandos. Así las respuestas transcripcionales que se puedan producir por acción de la VitD en los distintos tejidos vendrán dadas por el tipo de correguladores implicados, las cantidades relativas de los mismos, su manera de actuación y el conjunto de factores con los que interaccionen en los complejos transcripcionales. Se ha descrito que factores como TBL1 y TBLR1, presentes en los complejos correpresores formados por SMRT y NCoR son requeridos para la activación transcripcional por los NRs en presencia de ligando debido a su capacidad de mediar el intercambio de correpresores/coactivadores a través del reclutamiento del complejo ubiquitina/proteosoma 19S (148). Estas observaciones plantean que la unión de un ligando a los NRs podría hacer que los propios correpresores tuviesen funciones activadoras de la transcripción en determinados contextos donde el intercambio de factores durante el proceso de activación/represión génica así lo requiriera. Nuestros resultados en ensayos ChIP en los que la máxima presencia de la histona H4 acetilada en el promotor del gen *cyp*24 coincide con el máximo reclutamiento de SMRT, NCoR y HDAC3 claramente muestran que

en una población de células y de moléculas de DNA, la VitD puede provocar al mismo tiempo tanto una activación transcripcional como un detectable reclutamiento de correpresores al promotor diana. Estos datos no son más que el reflejo del equilibrio y el constante intercambio entre los estados de activación y de represión transcripcional donde probablemente cada factor implicado tenga una dualidad de acción pudiendo activar o reprimir dependiendo del conjunto de cofactores con los que esté interaccionando en ese momento particular.



Modelo simplificado de reclutamiento de SMRT y NCoR al heterodímero VDR/RXR en respuesta a Vitamina D. El heterodímero en ausencia de ligandos no une correpresores. La unión de un agonista al VDR desenmascararía la superficie de interacción de correpresores en el RXR mediante un cambio alostérico, produciéndose el reclutamiento de SMRT o NCoR al heterodímero. En este proceso es necesario la H12 del VDR. La unión del ligando del RXR o la mutación de residuos pertenecientes a la superficie de interacción con correpresores del mismo bloquearían el reclutamiento de estos correpresores. En este modelo no se incluye el papel inhibidor de la unión de correpresores que ejerce la H12 del RXR. CoR: correpresor; Mut H3 - H5: mutación de la superficie de interacción con correpresores.

3. Caracterización de distintos análogos de la vitamina D con actividad "disociada" y específica de tipo celular

La VitD juega un papel clave en procesos de diferenciación, proliferación, modulación del sistema inmune y en el mantenimiento de la homeostasis de minerales (41). Aunque su principal función parece ser el mantenimiento de la homeostasis del calcio (29), se han descrito importantes efectos diferenciadores e inhibitorios de la proliferación celular en diversos tipos de cáncer, como colon, próstata, mama y leucemias de células T. Además se han propuesto posibles acciones terapéuticas de los agonistas del VDR en casos de fallo renal, raquitismo, osteoporosis, psoriasis, y algunas enfermedades autoinmunes (135). Sin embargo, las dosis terapéuticas de VitD normalmente traen asociado un indeseable efecto hipercalcémico. Por ello, a lo largo de estos años se han tratado de sintetizar análogos de la VitD que carezcan de esos efectos secundarios. En esta parte del trabajo hemos analizado el comportamiento tanto *in vitro* como en células en cultivo de 7 derivados sintéticos de la VitD, que en su mayoría difieren del compuesto natural en distintas modificaciones de su cadena lateral. Los valores de calcemia medidos para estos compuestos fueron en todos los casos bastante menores que los del ligando natural, salvo en dos casos, los de los compuestos ZK161422 y ZK157202, que presentaban valores similares a la VitD o incluso superiores en el caso de ZK157202.

Analizamos el potencial agonista de estas sustancias mediante digestión limitada con proteasas y encontramos compuestos con escasa actividad agonista tomando como modelo el comportamiento del agonista parcial ZK159222 previamente caracterizado (27). ZK136607, ZK168492, ZK191732 y ZK168289 estabilizaban una conformación del receptor para la cual la H12 se encuentra desplazada y no permite la interacción con coactivadores. Además estos compuestos no promovían la interacción del receptor con coactivadores ni en solución ni sobre DNA, a excepción de ZK136607 que causaba unión de coactivadores en presencia de un elemento DR3. Esta falta de interacción eficaz con coactivadores explicaría la escasa potencia transactivadora de estos compuestos en promotores que contienen VDREs.

Con estos mismos criterios identificamos un compuesto con actividad superagonista, el ZK157202, tomando como modelo el comportamiento del potente agonista ya descrito ZK161422 (84). ZK157202, estabilizaba conformaciones agonísticas del receptor, promovía reclutamiento de coactivadores tanto en solución como sobre DNA y estimulaba la transcripción de promotores que contienen VDREs incluso con una potencia mayor que la propia VitD.

Podría pensarse que la distinción entre compuestos de naturaleza agonista o antagonista residiría en la capacidad de estimular la unión de los heterodímeros VDR/RXR al DNA (40). Sin embargo, nuestros resultados indican que la VitD y los análogos estudiados incrementan en igual medida la unión de VDR/RXR sobre un DR3. Así pues, la incapacidad de formación de heterodímeros estables sobre el DNA no parece ser la principal razón para la escasa actividad transactivadora de algunos de estos ligandos. Más bien parece ser la falta de unión de coactivadores al heterodímero lo que explique este comportamiento. Se ha propuesto que compuestos con capacidades superagonistas actuarían estabilizando durante más tiempo que el ligando natural las conformaciones agonísticas del receptor (28).

Esto podría explicar la mayor capacidad de los superagonistas ZK161422 y ZK157202 promoviendo el reclutamiento de coactivadores tanto en solución como sobre DNA. Por otro lado, su mayor potencia transactivadora podría deberse también a la diferente manera en que son metabolizados. Se ha descrito que ZK157202 no es metabolizado a través de la oxidación en el carbono 24, sino que es metabolizado por rutas alternativas y por diferentes isoformas del citocromo p450 (175). Esto le permitiría permanecer durante más tiempo en la célula y por tanto, presentar una mayor actividad que la VitD o que el derivado ZK161422.

Parte de los compuestos analizados presentaban respuestas transcripcionales dependientes de tipo celular. Algunos análogos, como ZK136607 y ZK168492, presentaban mayor actividad agonista en Hela que en 293T, y otros como ZK191732 y ZK168289 eran más activos en 293T. A su vez, la capacidad de estos compuestos para antagonizar la acción de la VitD dependía del contexto celular. La capacidad de estos análogos de mediar distintas respuestas transcripcionales en distintos tipos celulares los identifica como moduladores del VDR (VDRMs). Se ha propuesto que esta propiedad podría venir dada por un reclutamiento selectivo de cofactores en los distintos ambientes celulares (90). Debido a que los distintos ligandos producen diferentes conformaciones en el receptor, esta variación en las conformaciones podría llevar a la unión distintos cofactores en cada caso. Muy recientemente se han identificado ligandos de tipo no secosteroideo para el VDR con potentes efectos agonistas en queratinocitos, osteoblastos y células mononucleares de sangre periférica, pero con escasa actividad en células intestinales (123). Estos ligandos también presentan diferencias entre si en el reclutamiento del coactivador PGC-1. También se han observado diferencias en la unión de distintos miembros de los coactivadores p160 para análogos de tipo secosteroideo (190). En nuestro caso, los patrones de reclutamiento para los distintos coactivadores ensayados p160 o DRIP205, son similares pese a ser análogos de tipo secosteroiodeo. No obstante, puede ser que exista un reclutamiento diferencial de otros correguladores que hayan quedado fuera de nuestro análisis. La diferencia en la respuesta provocada por estos compuestos en unos u otros tejidos también podría radicar en la manera en que los mismos son metabolizados en cada tipo celular. Podrían existir variaciones en las vías metabólicas que predominan en los distintos tejidos o un patrón diferencial de expresión de enzimas implicadas en el metabolismo de estos compuestos que provocara que fuesen transformados a formas más o menos activas o simplemente degradados a distinta velocidad, explicando las diferencias de actividad entre distintos tipos celulares.

Una de las observaciones más interesantes de este trabajo es la característica que presentan algunos de estos compuestos conocida como actividad transcripcional "disociada". Pese a tener escasa potencia transactivadora, son capaces de transrreprimir con una potencia similar a la del ligando natural. Curiosamente estos ligandos, ZK136607, ZK159222, ZK168492 y ZK191732, presentan además muy poca actividad calcémica, lo que crea interesantes perspectivas desde el punto de vista terapéutico. En el caso del promotor RARβ2, los heterodímeros VDR/RXR se unirían a su elemento RARE con alta afinidad, aunque serían transcripcionalmente inactivos (92). El heterodímero VDR/RXR podría causar transrrepresión al interferir con la unión de RAR/RXR a su elemento. Estos derivados estimulan la unión de heterodímeros VDR/RXR sobre el DNA con una potencia similar a la de la VitD, lo que

explicaría que posean un efecto transrrepresor similar al del ligando natural. Otra posibilidad para explicar este mecanismo sería el secuestro de coactivadores por parte del heterodímero VDR/RXR en detrimento de RAR/RXR. Se ha comprobado que para que exista la transrrepresión de RARβ2 por VitD es necesaria la integridad del dominio AF-2 del VDR (93), lo que parecería implicar al reclutamiento de coactivadores en este fenómeno. Sin embargo, los compuestos ZK136607, ZK159222, ZK168492 y ZK191732, pese a que son capaces de transrreprimir, no presentan un reclutamiento de coactivadores efectivo, lo que hace pensar que puedan ser otros coactivadores aún no identificados los que estén implicados en este fenómeno, o bien que este mecanismo de transrrepresión no radique en la competición por coactivadores.

Analizando la transrrepresión de estos derivados de la VitD sobre los sitios AP-1 del promotor de la colagenasa nuevamente encontramos que ligandos con perfiles escasamente agonistas en transactivación, se comportan con tanta potencia como el agonista natural en transrrepresión. De nuevo todo parece indicar que los mecanismos moleculares que rigen la actividad transactivadora difieren de los que median la transrrepresión por estos compuestos. Aunque ya se habían descrito ligandos con actividad "disociada" en el caso de GR (200) y RAR (16, 39, 56, 67, 117), el que estas sustancias sean incapaces de estimular la transcripción eficientemente pero presenten un claro efecto anti AP-1 es un hallazgo nuevo para los ligandos de VDR. Ya que los complejos AP-1 regulan la expresión de genes implicados en procesos de proliferación y transformación oncogénica (3, 83, 115, 221), la identificación de compuestos capaces de reprimir la actividad AP-1 sin tener efectos transcripcionales no deseados en otros contextos tiene un gran interés desde un punto de vista terapéutico. Además de presentar efectos disociados, la capacidad transrrepresora de estos análogos depende del tipo celular. Así ZK136607 transrreprime el promotor de RARβ2 con más potencia que la VitD en Hela , pero no en 293T y los efectos de varios análogos sobre los sitios AP-1 del promotor de la colagenasa son mayores en 293T que en Hela. Esto nuevamente nos indica que el metabolismo que sufran estos compuestos en cada tipo celular puede jugar un papel decisivo en las respuestas transcripcionales resultantes.

Hemos visto como el agonista parcial ZK159222 era capaz de reclutar coactivadores y estimular la transcripción de promotores que contienen VDREs cuando se combinaba con el ligando del RXR. La combinación de estos análogos con el 9-*cis*RA produce una importante unión de coactivadores en los compuestos que por si solos no presentan un reclutamiento significativo de estos cofactores, y en el caso de los superagonistas ZK161422 y ZK157202, la adición del ligando del RXR produce un efecto sinérgico en el reclutamiento similar al de la VitD (datos no mostrados). Además estos análogos cooperan con el 9-*cis*RA presentando respuestas transcripcionales similares a las producidas por la VitD en el caso de los análogos de perfil antagonista y aún mayores que las obtenidas por la vitamina combinada con 9-*cis*RA en el caso de los superagonistas descritos (datos no mostrados). Así se abren aún mayores perspectivas para la aplicación terapéutica de estos compuestos, ya que además de presentar efectos disociados en cuanto a la activación y a la represión transcripcional, y respuestas dependientes de tejido, la combinación con el 9-*cis*RA incrementa las posibilidades de regulación de la señalización por este tipo de ligandos.

Conclusiones

Conclusiones

1. La estimulación de la transcripción del gen de la prolactina en respuesta a la T3 y al 9-*cis*RA, está mediada por un elemento de respuesta común, localizado en el "enhancer" distal del gen.

2. El ligando del RXR tiene un papel activo en los heterodímeros TR/RXR y VDR/RXR promoviendo el reclutamiento de coactivadores y la activación de la transcripción. Esto desecha la vieja idea de que el RXR actúa como un "compañero silencioso" en los heterodímeros formados con TR y VDR.

3. La combinación del 9-*cis*RA con los ligandos de sus parejas recupera el reclutamiento de coactivadores y la respuesta transcripcional en heterodímeros defectivos que contienen mutaciones puntuales en el dominio AF-2 de TR o VDR, restaura la unión de coactivadores mutantes a los heterodímeros, y confiere una fuerte capacidad agonista a ligandos inactivos. Se demuestra así la importante función del RXR en la señalización mediada por TR y VDR.

4. El 9-*cis*RA es capaz de potenciar la estimulación de la expresión del gen, *cyp24* por la vitamina D. Además coopera con distintos ligandos de VDR en un proceso fisiológico complejo como es la diferenciación de células de cáncer de colon mediada por el incremento de la expresión de E-Cadherina.

5. Análogos de la vitamina D con un perfil escasamente agonista y acciones dependientes de tipo celular, son capaces de transrreprimir con similar potencia al ligando natural. Su débil actividad calcémica, sugiere que la utilización de estos ligandos con "actividad disociada" pudiera ser de gran utilidad terapéutica.

6. El heterodímero VDR/RXR recluta los correpresores SMRT y NCoR en respuesta a vitamina D. Esto supone un nuevo modelo de unión de estos correpresores a los receptores nucleares en el cual el reclutamiento se produce por la unión de un ligando agonista. Este proceso además ocurre de manera cíclica sobre el promotor de un gen natural diana de la vitamina D.

7. La unión de un agonista al VDR es capaz de promover el reclutamiento de SMRT y NCoR a su pareja el RXR, para lo que se requiere el dominio AF-2 del VDR. Esto demuestra la intensa comunicación alostérica que se produce en el seno de estos heterodímeros y nuevamente, el importante papel que el RXR desempeña en los mismos. Además, la unión del ligando de RXR, sea agonista o antagonista, revierte el reclutamiento de correpresores.

8. El reclutamiento de SMRT y NCoR en respuesta a vitamina D regula negativamente la activación transcripcional por esta vitamina. Así el incremento de la unión de correpresores debido a la deleción del dominio AF-2 del RXR se traduce en una disminución de la activación transcripcional y la disminución de los niveles de SMRT o NCoR por técnicas de siRNA, en un aumento de la respuesta a la vitamina.

9. La abundancia relativa de coactivadores y correpresores en los distintos tipos celulares determina tanto la permisividad para la acción del ligando del RXR en el heterodímero TR/RXR, como la magnitud de las respuestas transcripcionales en los heterodímeros TR/RXR y VDR/RXR. Así las respuestas transcripcionales producidas por este tipo de heterodímeros vendrán determinadas en gran medida por el ambiente celular en el cual se desarrollan.

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